UNIVERSIDADE ESTADUAL DE CAMPINAS

Instituto de Biologia

Melissa de Oliveira Santos Garcia

ESTUDO DA DIVERSIDADE GENÉTICA E DA TAXA DE CRUZAMENTO EM *Stylosanthes* spp. ATRAVÉS DE MARCADORES MICROSSATÉLITES

Tese apresentada ao Instituto de Biologia para obtenção do título de Doutor em Genética e Biologia Molecular na área de Genética Vegetal e Melhoramento

Este exemplar corresponde à redação final da tese defendida pelo(a) candidato (a) Melissa de Oliveira Santos Garcia e aprovada pela Comissão Julgadora.

Orientadora: Profa. Dra. Anete Pereira de Souza

Co-orientadora: Dra. Rosângela M. S. Resende

Campinas, 2009

FICHA CATALOGRÁFICA ELABORADA PELA
BIBLIOTECA DO INSTITUTO DE BIOLOGIA – UNICAMP

Sa59e

Santos-Garcia, Melissa de Oliveira
Estudo da diversidade genética e da taxa de cruzamento em *Stylosanthes* spp. através de marcadores microssatélites / Melissa de Oliveira Santos Garcia. – Campinas, SP: [s.n.], 2009.

Orientadores: Anete Pereira de Souza, Rosângela Maria Simeão Resende.
Tese (doutorado) – Universidade Estadual de Campinas, Instituto de Biologia.

1. Microssatélites (Genética). 2. Plantas – Melhoramento genético. 3. Plantas forrageiras. 4. Leguminosa. I. Souza, Anete Pereira de. II. Resende, Rosângela Maria Simeão. III. Universidade Estadual de Campinas. Instituto de Biologia. IV. Título.

Título em inglês: Genetic diversity and mating system in *Stylosanthes* spp. as revealed by microsatellite markers.
Palavras-chave em inglês: Microsatellite (Genetics); Plant breending; Forage plants; Legumes.
Área de concentração: Genética vegetal e melhoramento.
Titulação: Doutora em Genética e Biologia Molecular.
Banca examinadora: Anete Pereira de Souza, Eliana Regina Forni-Martins, Flávio Antonio Maës dos Santos, Maria Lúcia Carneiro Vieira, Cláudio Takao Karia.
Data da defesa: 30/10/2009.
Programa de Pós-Graduação: Genética e Biologia Molecular.

Campinas, 30 de outubro de 2009.

Banca Examinadora

Profa. Dra. Anete Pereira de Souza (Orientadora) _____

Profa. Dra. Eliana Regina Forni Martins _____

Prof. Dr. Flávio Antonio Maës dos Santos _____

Prof. Dra. Maria Lúcia Carneiro Vieira _____

Dr. Cláudio Takao Karia _____

Prof. Dr. Cláudio Lopes de Souza Júnior _____

Prof. Dr. Michel Georges Albert Vincentz _____

Dr. Marcelo Mattos Cavallari _____

Dedico

À memória de meu avô, **Luiz**,

Pelo exemplo.

Ofereço

Aos meus pais **Aparecida** e **Wilson**,

À minha irmã **Larissa**,

Ao meu marido **Alexandre**,

Pelo carinho e apoio.

Agradeço

À Fundação de Amparo à Pesquisa do Estado de São Paulo (Fapesp), pelo apoio financeiro no projeto e pela bolsa concedida.

À minha orientadora Profa. Dra. Anete Pereira de Souza, por ter confiado em mim em todos os momentos da realização desse projeto e pela capacidade em conseguir mostrar que as coisas são mais simples do que parecem e que tudo tem solução.

À minha co-orientadora Dra. Rosângela M. S. Resende e a todos da Embrapa Gado de Corte, pela ajuda durante o desenvolvimento do projeto.

Aos pesquisadores da Embrapa Cerrados, especialmente ao Dr. Cláudio T. Karia e ao Dr. Fábio G. Faleiro, por disponibilizarem material para análise.

À colaboradora Dra. Maria Imaculada Zucchi, pela ajuda na análise dos dados e pela paciência.

Ao Prof. Dr. Michel Vincentz, por ter disponibilizado seu laboratório para que parte dos experimentos pudesse ser realizada.

Aos membros da pré-banca, Profa. Dra. Maria Lúcia Carneiro Vieira e Prof. Dr. Michel Vincentz, pela disponibilidade e ajuda na pré-avaliação da tese.

Aos membros da banca de defesa, pela disponibilidade em contribuir para que esse trabalho fosse melhorado.

A todos os técnicos que passaram pelo laboratório durante esses quase cinco anos, principalmente à Patty, pelas inúmeras acrilamidas e colorações de prata.

Ao Ju que, além de resolver qualquer problema técnico, faz um ótimo café e sempre me diverte.

Aos alunos de I.C., Sassaki e Thaís, pela ajuda fundamental na bancada e por me darem a chance de aprender mais.

Aos colegas do lab, por tornar o trabalho mais fácil e divertido. Em especial à Pri, por ter me acolhido quando cheguei e por ter se mantido ao meu lado sempre que precisei. Ao Thi e à Fer, pelas conversas no café e pelo cuidado com a minha alimentação nos últimos meses. À Lí, por me divertir e por me lembrar tanto a minha família, com seu jeito extremamente espontâneo.

À minha mãe Aparecida, por ter me encorajado a vir para Campinas, por ter se mantido ao meu lado em todas as minhas escolhas, por sempre confiar que tudo ia dar certo e por me ajudar a acreditar nisso.

Ao Lê, meu marido querido, por acreditar em mim mais do que eu mesma acredito, por tentar me acalmar sempre que me desesperei e por estar ao meu lado sempre, mesmo quando estávamos a um mundo de distância.

Sumário

Prefácio 13

Resumo 15

Abstract 17

1 Introdução 19

2 Revisão de Literatura 22

2.1 O Gênero *Stylosanthes* Sw. 22

2.2 Marcadores Moleculares em Plantas 28

2.3 Uso de Marcadores Microssatélites na Caracterização de Bancos de Germoplasma 33

2.4 Sistema de Cruzamento em Plantas 37

2.5 Marcadores Moleculares em *Stylosanthes* Sw. 40

3 Objetivos 47

4 Artigo I 48

5 Artigo II 52

6 Artigo III 85

7 Artigo IV 91

8 Artigo V 114

9 Artigo VI 119

10 Conclusões 144

10.1 Desenvolvimento dos Marcadores Microssatélites 144

10.2 Estudo da Taxa de Cruzamento 145

10.3 Estudo da Diversidade Genética em Bancos de Germoplasma 145

11 Referências Bibliográficas 147

Prefácio

Os resultados obtidos durante esse trabalho de Doutorado estão apresentados na forma de seis artigos. O primeiro intitulado "Isolation and characterization of microsatellite loci in tropical forage *Stylosanthes capitata* Vogel", publicado na Molecular Ecology Resources (9, 192-194, 2009), trata do desenvolvimento de oito marcadores microssatélites polimórficos para a espécie *S. capitata* Vog. e da sua transferibilidade para as espécies *S. guianensis* (Aubl.) Sw. e *S. macrocephala* Ferr. et Costa. O segundo artigo "Tropical Forage Legumes: I Estimation of the outcrossing rate of important *Stylosanthes* species" será submetido à revista Theoretical and Applied Genetics e trata do desenvolvimento de mais 15 microssatélites polimórficos para *S. capitata* Vog. e da estimação da taxa de cruzamento em *S. capitata* e *S. guianensis*. O terceiro artigo, intitulado "Polymorphic microsatellite loci for *Stylosanthes macrocephala* Ferr. et Costa, a tropical forage legume." foi publicado na Conservation Genetics Resources (Online first, DOI 10.1007/s12686-009-9112-x) e mostra o desenvolvimento de 13 marcadores microssatélites polimórficos para a espécie *S. macrocephala* Ferr. et Costa, assim como sua transferência para as espécies *S. capitata* Vog., *S. guianensis* (Aubl.) Sw. e *S. pilosa.* O quarto artigo, intitulado "Tropical Forage Legumes: II Genetic diversity in *Stylosanthes macrocephala* Ferr. et Costa", será submetido à revista Theoretical and Applied Genetics e apresenta um estudo de diversidade genética em 134 acessos de *S. macrocephala* Ferr. et Costa, pertencentes à Embrapa Cerrados, através da utilização dos 13 marcadores microssatélites

desenvolvidos para essa espécie. O quinto artigo, "Isolation and characterization of microsatellite loci in the tropical forage legume *Stylosanthes guianensis* (Aubl.) Sw." foi publicado na Conservation Genetics Resources (Online first, DOI 10.1007/s12686-009-9010-2, 2009) e trata do desenvolvimento de 20 marcadores microssatélites polimórficos para *S. guianensis* (Aubl.) Sw. e da sua transferibilidade para as espécies *S. capitata* Vog., *S. macrocephala* Ferr. et Costa e *S. pilosa* Ferr. et Costa. O sexto artigo, "Tropical Forage Legumes: III Genetic diversity in *Stylosanthes guianensis* (Aubl.) Sw." será submetido à revista Theoretical and Applied Genetics e trata da utilização dos 20 locos polimórficos desenvolvidos para *S. guianensis* (Aubl.) Sw. em um estudo da diversidade genética de 150 acessos pertencentes ao banco de germoplasma da Embrapa Cerrados.

Resumo

No Brasil, a pecuária bovina é baseada principalmente na utilização de pastagens para alimentação animal, sendo a maioria destas cultivadas. Gramíneas do gênero *Urochloa* P. Beauv. originárias da África Setentrional têm sido as mais utilizadas pelos pecuaristas, juntamente com algumas leguminosas de origem sul-americana. Os maiores problemas enfrentados pelos produtores são a degradação das pastagens e o seu alto custo de recuperação, principalmente pela necessidade de uso de fertilizantes químicos nitrogenados. Devido à capacidade das leguminosas de transformar o nitrogênio atmosférico e fixá-lo no solo, seu uso consorciado a gramíneas pode ser indicado como uma alternativa menos onerosa para recuperação das pastagens. Dentre as leguminosas já testadas como pastagens, as do gênero *Stylosanthes* Sw. têm se mostrado passíveis de utilização em regiões de solos de baixa fertilidade, apresentando bons resultados quando consorciadas a gramíneas. O Brasil é o maior centro de origem e diversidade deste gênero, com ocorrência das espécies de maior potencial forrageiro, havendo vários acessos disponíveis em bancos de germoplasma. Para que os programas de melhoramento possam utilizar de forma adequada os bancos de germoplasma existentes, é necessário um conhecimento da quantidade e da distribuição da diversidade genética dentro dessas coleções. Além disso, para grande parte das espécies do gênero, as taxas de cruzamento são desconhecidas ou foram estimadas com base em caracteres morfológicos, que podem sofrer influência do ambiente e são encontrados em número restrito. Nesse contexto, no

presente trabalho foram desenvolvidos marcadores microssatélites para três espécies do gênero *Stylosanthes* Sw. (*S. capitata* Vog., *S. guianensis* (Aubl.) Sw. e *S. macrocephala* Ferr. et Costa). Devido à disponibilidade dos experimentos, foi possível utilizar os microssatélites desenvolvidos para estimar a taxa de cruzamento de *S. capitata* Vog. e *S. guianensis* (Aubl.) Sw., mostrando que ambas as espécies apresentam um sistema misto de reprodução com predominância de autogamia. Considerando que as flores de *Stylosanthes* são cleistógamas, a taxa de cruzamento encontrada foi alta (26% para *S. guianensis* (Aubl.) Sw. e 31% para *S. capitata* Vog.) e deve ser considerada durante a multiplicação de sementes para manutenção do banco de germoplasma, a fim de manter a integridade individual de cada acesso. Foi também observada uma variação da taxa de cruzamento entre diferentes progênies. Também foi avaliada a diversidade genética em bancos de germoplasma de duas espécies do gênero (*S. guianensis* (Aubl.) Sw. e *S. macrocephala* Ferr. et Costa), mostrando a formação de grupos bem definidos entre os acessos dessas espécies e que podem ser considerados durante o melhoramento. Em *S. guianensis* (Aubl.) Sw., que apresenta uma classificação taxonômica controversa, os grupos formados estão de acordo com um dos modelos taxonômicos propostos. Os microssatélites desenvolvidos, assim como os dados de taxa de cruzamento e de diversidade genética gerados nesse trabalho, são um passo em direção a um melhor entendimento do gênero e uma ferramenta para que novos trabalhos possam ser realizados.

Abstract

In Brazil, most of the cattle are grown in cultivated pastures. Forage grasses belonging to the African genus *Urochloa* P. Beauv. have been the most commonly used, together with some American legumes. Soil degradation and high costs for reclaiming are the major problems of pastures, since nitrogen enrichment is necessary. Considering that legumes can fix atmospheric nitrogen into soil, their use in consortium with grasses has been a plausible alternative for soil reclaiming. Among feasible legumes for pastures, those belonging to the genus *Stylosanthes* Sw. have proved to be suitable for low fertile soils and and for consortium with grasses. Brazil is the major center of origin and diversity of this genus, including its most promising species. Many *Stylosanthes* Sw. accessions are available in germplasm collections and their use could be potentiated by further knowledge on the available genetic diversity. In addition, there is still limited knowledge on the mating system of most of *Stylosanthes* Sw. species. In the present work, microsatellite markers were developed for *S. capitata* Vog., *S. guianensis* (Aubl.) Sw. and *S. macrocephala* Ferr. et Costa aiming at studying the genetic diversity and mating systems of *Stylosanthes* Sw. species. The microsatellite analysis on the mating system of *S. capitata* Vog. and *S. guianensis* (Aubl.) Sw. showed a mixed mating system with predominance of self-fertilization. Considering that *Stylosanthes* flowers are cleistogamous, the observed outcrossing rates were relatively high, being 26% for *S. guianensis* (Aubl.) Sw. and 31% for *S. capitata* Vog.. These outcrossing rates should be taken into consideration in

seed multiplication, for the purpose of maintaining the genetic integrity of individual accessions. Variation in the estimates of outcrossing among different progenies was observed. The microsatellite studies on the genetic diversity in *S. guianensis* (Aubl.) Sw. and *S. macrocephala* Ferr. et Costa demonstrated that there are genetic distinct groups within the germplasm collection that could be useful for breeding purposes. In *S. guianensis* (Aubl.) Sw., which has a controversial taxonomy, the observed groups were in agreement with one of the proposed taxonomical classification. The microsatellites developed for *Stylosanthes* Sw. species and the data on mating system and genetic diversity presented herein represent important knowledge and tools towards the understanding of this genus and are potentially useful for further studies.

1 Introdução

Nos cerrados brasileiros, a exemplo do que ocorre em outras regiões tropicais e subtropicais, a pecuária bovina é baseada principalmente na utilização de pastagens para alimentação animal, resultando no que é denominado "boi verde". No Brasil, cerca de 175 milhões de hectares são ocupados por pastagens, sendo cerca de 40% dessa área localizada na região centro-oeste, a qual mais se destaca na pecuária brasileira (Censo Agropecuário/ Instituto Brasileiro de Geografia e Estatística 2006). Dentre as várias opções de forrageiras tropicais, predominam as gramíneas e, dentro destas, as do gênero *Urochloa* P. Beauv., que respondem por 50% das sementes produzidas e por 85% das sementes comercializadas para formação de pastagens no Brasil central (Santos-Filho 1996).

Um dos maiores problemas enfrentados na pecuária de corte é a degradação das pastagens e o seu alto custo de recuperação, principalmente pela necessidade de uso de fertilizantes químicos nitrogenados, obtidos a partir do petróleo, recurso não-renovável, e de custo comercial elevado (Embrapa Gado de Corte 2000). Estima-se que cerca de 80% das pastagens cultivadas do Brasil Central, que respondem por 55% da produção de carne nacional, encontra-se em algum estágio de degradação (Macedo *et al*. 2000). O termo degradação da pastagem pode ser definido como sendo o processo evolutivo da perda de vigor, da produtividade, da capacidade de recuperação natural para sustentar os níveis de produção e da capacidade de superar os efeitos nocivos de pragas e doenças (Macedo & Zimmer 1993). A causa da degradação

está relacionada a fatores como: maneira de preparo do solo, escolha de espécies forrageiras, fertilidade do solo, ataque de pragas, uso do fogo, ausência de consorciações, manejo inadequado do pastoreio, dentre outros (Franco & Rosa 2003).

O uso de leguminosas consorciadas a gramíneas pode reduzir os investimentos em insumos agrícolas e os impactos ambientais que levam à degradação das pastagens. Isso é possível graças à capacidade das leguminosas de transformar o nitrogênio encontrado na atmosfera e fixá-lo biologicamente no solo através de simbiose com bactérias do gênero *Rhizobium*. Além disso, as leguminosas também possibilitam um maior ganho de peso nos animais devido à grande quantidade de proteína presente em suas partes aéreas (Embrapa Gado de Corte 2000). A diversidade de espécies de leguminosas é grande, sendo que, dependendo da região, algumas espécies mostram-se melhores para o cultivo. O gênero *Stylosanthes* Sw. tem se mostrado passível de utilização em solos pobres como os do cerrado, contribuindo positivamente para o sistema de produção (Grof *et al*. 1979).

O Brasil é o principal centro de origem e diversidade do gênero *Stylosanthes* Sw., com ocorrência de 45% das 48 espécies, entre elas as de maior potencial forrageiro (Costa 2006). Estudos das espécies do gênero *Stylosanthes* Sw. visando sua utilização como forrageiras no Brasil iniciaram-se na década de 30, sendo que os programas de melhoramento tiveram início apenas na década de 60 e basearam-se na avaliação dos numerosos acessos

coletados, seleção dos mais adaptados e realização de misturas de acessos para lançamento como cultivares (Andrade & Karia 2000).

Com o avanço rápido da biologia molecular, um grande número de técnicas desenvolvidas tem se mostrado útil em programas de melhoramento, como os marcadores moleculares, que permitem identificar origem parental, identificar genótipos, quantificar variabilidade genética, estimar taxas de cruzamento, dentre outras aplicações (Prasad *et al*. 2000, Reusch 2000). Neste contexto, este trabalho visou estudar a diversidade genética e o sistema de cruzamento em espécies importantes de *Stylosanthes* Sw. através do desenvolvimento de marcadores microssatélites, gerando informações que podem ser úteis para os programas de melhoramento e para o manejo dos bancos de germoplasma deste gênero.

2 Revisão de Literatura

2.1 O Gênero *Stylosanthes* Sw.

O gênero *Stylosanthes*, estabelecido por Swatz em 1788, é um membro da família Fabaceae, subfamília Papilionoideae, tribo Aeschynomeneae, subtribo Stylosanthineae. Este gênero contém 48 espécies, distribuídas nas regiões tropicais e subtropicais das Américas, na África e no sudeste da Ásia (Costa & Ferreira 1984, Costa 2006). O gênero apresenta dois centros de diversidade, sendo o principal no Brasil central, onde são encontradas 45% das espécies e são observados o maior endemismo e variação fenotípica (Costa 2006). O outro centro de diversidade encontra-se na região do México e do Caribe, na América Central (Stace & Cameron 1984).

Duas seções subgenéricas, seção Stylosanthes Vog. e seção Styposanthes Vog. têm sido reconhecidas de acordo com caracteres morfológicos (Kirkbride & Kirkbride 1985). O número cromossômico básico do gênero é x=10, havendo espécies diplóides (2n=40) como *S. viscosa* (L.) Sw., *S. guianensis* (Aubl.) Sw. e *S. macrocephala* Ferr. et Costa, espécies tetraplóides como *S. scabra* Vog. e *S. capitata* Vog. e, ainda, uma espécie hexaplóide, *S. erecta* P. Beauv. (Stace & Cameron 1984, Vieira *et al*. 1993). Enquanto espécies diplóides são encontradas em ambas as seções, espécies poliplóides (2n=40 e 2n=60) são restritas à seção Styposanthes Vog. e supõe-se que sejam produtos de hibridação entre diplóides pertencentes a diferentes seções, seguida de poliploidização (Stace & Cameron 1984). Como exceção para esta suposta origem das espécies poliplóides, está a espécie *S. capitata*

Vog., que foi provavelmente originada pela hibridação entre as espécies diplóides *S. pilosa* Ferr. et Costa e *S. macrocephala* Ferr. et Costa, ambas da seção Styposanthes (Liu *et al*. 1999, Gillies & Abbott 1996).

A identificação de espécies em *Stylosanthes* Sw. baseada em caracteres morfológicos é controversa, sendo que diferentes pontos de vista existem sobre os conceitos de espécie e sobre quais caracteres são mais estáveis e devem ser utilizados na sua identificação (Costa & Ferreira 1984, t' Manetje 1984). Com avanço de técnicas moleculares, muitos trabalhos vêm sendo realizados com o objetivo de elucidar as relações interespecíficas dentro do gênero (Vieira *et al.* 1997, Liu *et al.* 1999, Vander Stappen *et al*. 1999a), sendo que um maior interesse em *Stylosanthes* Sw. surgiu, principalmente, após a descoberta de seu potencial para melhorar as condições das pastagens (Sawkins *et al*. 2001). Desde então, algumas espécies vêm se destacando como leguminosas forrageiras para as condições do cerrado, por apresentarem alto rendimento de matéria seca, crescerem em solos relativamente pobres e ácidos e serem capazes de incorporar nitrogênio ao solo (Resende *et al*. 2008).

Além da sua utilização como forrageira, *Stylosanthes* Sw. tem potencial para utilização em diversos outros sistemas de produção incluindo sistemas agroflorestais e silvipastoris, conservação e recuperação de solos e criação de bancos de proteínas (Barcellos *et al.* 2001, Cameron & Chakraborty 2004, Pathak *et al*. 2004). No Brasil, três espécies são consideradas mais promissoras para utilização como pastagem e também nos outros sistemas supramencionados, *S. capitata* Vog., *S. guianensis* (Aubl.) Sw. e *S.*

macrocephala Ferr. et Costa (Cameron & Chakraborty 2004, Andrade *et al*. 2004).

S. capitata Vog. (2n=40) (Figura 1A) está distribuída no Brasil e na Venezuela e é uma planta perene, subarbustiva, com hábito variando de prostrado a ereto . Produz grande quantidade de sementes e apresenta elevados rendimentos de matéria seca, sendo que suas inflorescências apresentam alto valor nutritivo (Williams *et al.* 1984, Costa 2006).

S. guianensis (Aubl.) Sw. (2n=20) (Figura 1B) é a espécie de *Stylosanthes* Sw. com a mais ampla distribuição geográfica, apresentando grande diversidade fenotípica (Williams *et al.* 1984; Vieira *et al*. 1993). Em conseqüência, sua classificação taxonômica é bastante controversa, sendo que diferentes classificações são propostas com base em diferentes caracteres morfológicos. t' Manetje (1984) reconheceu sete variedades dentro da espécie, enquanto Ferreira & Costa (1979) consideraram essas variedades como diferentes espécies e dividiram o que t' Manetje (1984) considerava como *S. guianensis* var. *guianensis* em três novas variedades. Mais tarde, uma quarta variedade foi descrita (Ferreira *et al.* 1985). Costa (2006) reafirmou a subdivisão de *S. guianensis* (Aubl.) Sw. em quatro variedades, *S. guianensis* var. *guianensis*, *S. guianensis* var. *canescens*, *S. guianensis* var. *microcephala* e *S. guianensis* var. *pauciflora*. As plantas dessa espécie são perenes, de herbáceas a arbustivas, cujos hábitos variam de prostado a ereto, sendo a variedade *microcephala* de hábito marcadamente prostrado (Costa 2006). *S. guianensis* (Aubl.) Sw. é considerada como uma das mais promissoras para utilização como forrageira no cerrado, sendo que vários acessos são os de mais

alta produção de matéria seca e retenção de folhas verdes em períodos de seca em solos de baixa fertilidade (Andrade & Karia 2000).

A espécie *S. macrocephala* Ferr. et Costa (2n=20) (Figura 1C) é uma planta perene, de herbácea a subarbustiva, de hábito geralmente prostrado (Costa 2006). É encontrada nas regiões central e leste do Brasil e apresenta baixa produção de matéria seca (Williams *et al.* 1984, Vieira *et al*. 1993).

Figura 1 – A – *S. capitata* Vog., B – *S. guianensis* (Aubl.) Sw., C – *S. macrocephala* Ferr. et Costa. Fotos: A, B – Dra. Rosângela M. S. Resende; C – Dr. Celso Dornelas.

No Brasil, assim como na América do Sul, o melhoramento de *Stylosanthes* Sw. tem se limitado às três espécies acima mencionadas. Para as condições brasileiras, foram recomendadas ou lançadas, até o momento, sete cultivares (Deodoro, DeodoroII, IRI 1022, Bandeirante, Pioneiro, Mineirão e Campo-Grande) e outra cultivar (Bela) encontra-se em fase de pré-lançamento (Andrade *et al.* 2004, Karia 2008).

S. guianensis var. *pauciflora* cv. Bandeirante é uma planta semi-ereta, com média de 0,65m de altura. Tem florescimento tardio e é bem adaptada a solos pobres, produzindo 80% da sua produção máxima em 60% de saturação de alumínio e em pH muito baixo. Essa cultivar foi recomendada para a região da Amazônia Ocidental e outras regiões úmidas. É tolerante à seca e apresenta uma tolerância moderada a sombreamento (Tropical forages 2005). Produz

cerca de 2500 Kg/ha ano de matéria seca, com teor médio de proteína bruta em torno de 12%. A produção de sementes é considerada baixa quando comparada às cultivares de origem Australiana, o que levou à pouca utilização dessa cultivar (Andrade & Karia 2000).

A cultivar Pioneiro pertence à espécie *S. macrocephala* Ferr. et Costa e foi coletada em Planaltina, DF em 1974. Tem uma altura média de 0,50 cm e mostrou resistência à antracnose em oito dos 11 locais onde foi avaliada, sendo recomendada para a região de cerrados. Apesar de apresentar grande potencial de utilização, tolerância à antracnose e relativa facilidade na produção de sementes, não despertou interesse dos produtores de sementes (Andrade & Karia 2000, Karia 2008, Tropical Forages 2005).

A cultivar Mineirão, *S. guianensis* var. *guianensis*, foi lançada pelas Embrapas Gado de Corte e Cerrados e é uma planta perene, semi-ereta e que atinge até 2,5m de altura. Apresenta boa tolerância à seca e ao frio e resistência à antracnose. Foi coletada no estado de Minas Gerais e seu consumo pelo gado é aumentado na época seca, quando ainda apresenta 12% de proteína na parte aérea. Em condição comercial, produz 30 a 60 Kg/ha de semente, mas pode produzir até 120 Kg/ha em condições irrigadas. Esta cultivar ainda é comercializada no Brasil, embora o preço de suas sementes seja considerado alto pelos pecuaristas (Tropical Forages 2005, Karia 2008).

A Embrapa Gado de Corte lançou a cultivar Campo-Grande, que é constituída por uma mistura física de sementes de duas espécies de *Stylosanthes* Sw., 80% de *S. capitata* Vog. e 20% de *S. macrocephala* Ferr. et Costa. As plantas foram coletadas em solos de baixa fertilidade, em uma

antiga área de um experimento de competição de acessos das duas espécies. *S. macrocephala* Ferr. et Costa tem um crescimento mais horizontal e as folhas mais pontiagudas, enquanto *S. capitata* Vog. apresenta um hábito de crescimento mais vertical e folhas mais arredondadas. Essa cultivar apresenta resistência à antracnose e sua produção de matéria seca, nas condições de Mato Grosso do Sul, atinge 14 t/ha ano, com teor de proteína de 12 a 18%. A produção de sementes também é alta, acima de 200Kg/ha, o que faz suas sementes terem um preço bem mais acessível do que as da cultivar Mineirão. Entretanto, a produção de forragem no período de seca é menor do que a da cultivar Mineirão, em regiões do Distrito Federal, norte de Goiás e Minas Gerais e no sudeste da Bahia (Embrapa Gado de Corte 2000, Karia 2008). A cultivar Campo- Grande é comercializada no Brasil e estima-se que sua área plantada seja de 160 a 200 mil hectares (Karia 2008).

A utilização mais abrangente de *Stylosanthes* Sw. apresenta como principais limitantes a susceptibilidade à antracnose, a baixa persistência de algumas variedades e a baixa produtividade (Phaikaew *et al*. 2004), cabendo aos programas de melhoramento, contornar esses limitantes. Para que o sucesso desses programas seja alcançado, é fundamental um maior conhecimento das características morfoagronômicas dos acessos e da sua utilização em diferentes regiões, assim como o conhecimento sobre a diversidade genética disponível aos melhoristas, nas coleções de germoplasma desse gênero.

2.2 Marcadores Moleculares em Plantas

Marcadores genéticos são quaisquer características, processos bioquímicos ou fragmentos de DNA que permitem a distinção de indivíduos geneticamente diferentes. Em plantas, quatro tipos de marcadores genéticos vêm sendo utilizados: morfológicos, citológicos, bioquímicos e moleculares (Borém & Miranda 2005).

Até meados da década de 60, os estudos de genética e melhoramento de plantas se baseavam em marcadores morfológicos, determinados por mutações simples em um gene em particular, gerando alterações fenotípicas de fácil identificação. Esses marcadores contribuíram significativamente para o desenvolvimento teórico da análise de ligação gênica e para a construção das primeiras versões de mapas genéticos em plantas. No entanto, o número reduzido de marcadores fenotípicos disponíveis, a ausência de ligação destes com caracteres de importância econômica, os efeitos deletérios das mutações e o fato de muitos marcadores só poderem ser identificados na fase adulta das plantas, limitaram sua utilização para a geração de mapas genéticos e para o melhoramento (Ferreira & Grattapaglia 1998, Guimarães & Moreira 1999).

O primeiro avanço nesse quadro iniciou-se com o desenvolvimento de marcadores isoenzimáticos, que possibilitaram a ampliação no número de marcadores genéticos disponíveis. Esses marcadores se baseiam na identificação de diferentes formas de uma mesma enzima, através de eletroforese ou de análises histoquímicas. As isoenzimas apresentam algumas desvantagens como o fato de poderem sofrer seleção, a existência de um

número restrito de locos e a dificuldade na sua interpretação (Ferreira & Grattapaglia 1998).

Com o avanço da biologia molecular, surgiram vários métodos de detecção de polimorfismo genético, diretamente na molécula de DNA. Teoricamente, qualquer fragmento de DNA pode ser utilizado como marcador molecular, desde que revele polimorfismo entre indivíduos (Souza 2001). As técnicas envolvendo marcadores moleculares vêm sendo cada vez mais aperfeiçoadas e automatizadas e seu desenvolvimento, juntamente com o avanço nas áreas de bioinformática e estatística, tem contribuído para o maior conhecimento sobre a estrutura genética de espécies cultivadas e silvestres (Guimarães & Moreira 1999).

Os primeiros marcadores de DNA a serem utilizados analisavam o polimorfismo no tamanho de fragmentos gerados pelo corte com enzimas de restrição, sendo conhecidos pela sigla RFLP's (*Restriction Fragment Lenght Polymorphism*). A técnica de RFLP é baseada na digestão do DNA genômico com enzimas de restrição, seguida da transferência dos fragmentos separados em um gel de agarose para membranas de nitrocelulose, onde são hibridizados com sondas de DNA espécie-específicas marcadas radioativamente. Esses marcadores foram os primeiros a serem empregados na construção de mapas genéticos em humanos (Botstein *et al.* 1980) e, posteriormente, foram amplamente aplicados em plantas, possibilitando a construção de mapas em muitas espécies cultivadas (Graner *et al.* 1991, Gardiner *et al*. 1993). Os marcadores RFLP's são codominates, ou seja, tanto os indivíduos homozigóticos quanto os heterozigóticos podem ser identificados na população.

Entre as limitações dessa técnica, estão a dificuldade de automação, já que envolve uma série de passos laboriosos, e o uso de sondas espécie-específicas radioativas.

Na década de 80, foi descrita a PCR (*Polymerase Chain Reaction*), que consiste na síntese *in vitro* de milhões de cópias de um segmento específico de DNA através da extensão, por meio de DNA polimerase, de um par de oligonucleotídeos, utilizados como iniciadores (*primers*) (Mullis & Faloona 1987). Sua facilidade, rapidez, versatilidade e sensibilidade possibilitaram o surgimento de uma nova geração de marcadores moleculares.

Os primeiros marcadores desenvolvidos com base em PCR foram os RAPD's (*Random Amplified Polymorphic DNA*). Esses marcadores utilizam um único *primer* curto, de sequência aleatória sendo que, para que um fragmento de DNA seja amplificado, duas regiões complementares ao *primer* devem estar separadas por até 2000 pb e em orientações opostas. São marcadores dominantes, já que o fragmento de DNA será ou não amplificado, impossibilitando a visualização de indivíduos heterozigóticos. Diferentes tamanhos de fragmento são gerados de acordo com a presença dos sítios complementares ao *primers* e esses fragmentos podem ser visualizados em géis de agarose (Williams *et al.* 1990). Por não necessitar do conhecimento prévio da sequência de DNA da espécie a ser estudada, ser de simples aplicação e necessitar de pequenas quantidades de DNA, os RAPD's foram extremamente utilizados nas mais diversas espécies de plantas e com as mais diversas aplicações (Newbury & Ford-Lloyd 1993, Virk *et al*. 1995). Porém esses marcadores perderam popularidade devido à baixa reprodutibilidade dos

experimentos, causada por diferenças na concentração de DNA utilizada, velocidade de mudança nas temperaturas durante os ciclos de amplificação, diferenças na concentração dos íons de magnésio e tipo de polimerase utilizada (Souza 2001).

Assim como os RAPD's, os AFLP's (*Amplified Fragment Lenght Polymorphism*) também não necessitam do conhecimento prévio da sequência de DNA e são de caráter dominante. Essa técnica combina características do RFLP e do RAPD, utilizando a alta especificidade dos sítios de restrição associada à rapidez e à informatividade da amplificação, via PCR, de fragmentos utilizando iniciadores aleatórios. A técnica consiste em uma digestão inicial do DNA genômico com uma determinada enzima de restrição, seguida de uma amplificação seletiva dos fragmentos gerados. Os fragmentos amplificados são separados em gel desnaturante de acrilamida e podem ser visualizados após coloração com prata ou através da marcação radioativa de um dos *primers* utilizados na amplificação seletiva, seguida de exposição a um filme autoradiográfico (Guimarães & Moreira 1999, Souza 2001). Por gerar uma quantidade grande de informação, esses marcadores também vêm sendo muito utilizados em plantas, com diferentes aplicações (Mackill *et al*. 1996, Zhu *et al*. 1998, Vuylsteke *et al*. 1999). Entretanto os AFLP's apresentam as deficiências de um marcador dominante, além da técnica ser cara e trabalhosa.

Microssatélites ou SSR's (*Simple Sequence Repeats*) são sequências de 2-6 pares de bases de comprimento repetidas em *tandem*. Estão presentes nos genomas de todos os organismos estudados até o momento, sendo encontrados de forma dispersa e em alta freqüência (Hamada *et al*. 1982,

Tautz & Renz 1984, Litt & Luty 1989). O número de repetições do microssatélite pode variar, sendo que sua sequência adjacente costuma ser conservada entre indivíduos diferentes da mesma espécie ou de espécies próximas, o que permite que o polimorfismo no número de repetições possa ser detectado, via PCR, através da utilização de *primers* específicos complementares às regiões adjacentes. Esse marcador apresenta como principais vantagens, ser abundante e amplamente distribuído nos genomas, codominante e multialélico. Sua principal desvantagem é a necessidade do conhecimento prévio da sequência de DNA da espécie a ser estudada. Porém com a redução dos custos de sequenciamento, esses marcadores se tornaram populares e vêm sendo desenvolvidos a partir de sequências genômicas ou expressas. Sua utilização em plantas é ampla, sendo utilizados tanto em estudos de populações naturais como em estudos mais aplicados em espécies cultivadas (Temnykh *et al.* 2000, Peleg *et al*. 2008, Kahrood *et al*. 2008).

Single nucleotide polymorphisms (SNPs) são variações na seqüência de DNA que ocorrem quando um único nucleotídeo na seqüência do genoma é alterado. Esses polimorfismos, juntamente com as deleções e inserções, são responsáveis pela maior parte da variação na maioria dos organismos (Cho *et al*. 1999, Rafalski & Tingey 2008) e são amplamente distribuídos pelo genoma, sendo mais abundantes em regiões não transcritas e em regiões que flanqueiam microssatélites (Mogg *et al*. 2002, Bundock & Henry 2004). Por serem abundantes em muitas espécies de plantas (Barker & Edwards 2009, Ganal *et al*. 2009), os SNPs têm sido muito utilizados como marcadores, criando perspectivas para o diagnóstico de doenças, a seleção assistida por

marcadores (MAS), a construção de mapas genéticos de alta resolução e a caracterização varietal (Batley *et al*. 2003), sendo considerados o sistema de marcadores mais atraente desenvolvido até o momento (Gupta *et al*. 2001). Suas principais vantagens residem na sua abundância e facilidade para automação, sendo sua principal desvantagem a necessidade de uma grande quantidade de sequências para que possam ser identificados.

Outra forma de acessar a variação na molécula de DNA consiste na amplificação de regiões conhecidas e na comparação de suas sequências, a fim de se estudar a história evolutiva em diferentes níveis taxonômicos. Essa metodologia, conhecida como filogenia molecular, tem auxiliado na elucidação das relações filogenéticas de muitos grupos de plantas através de estudos de genes nucleares (Álvarez & Wendel, 2003), cloroplastidiais (Horres *et al*. 2008) e mitocondriais (Palmer 1992).

2.3 Uso de Marcadores Microssatélites na Caracterização de Bancos de Germoplasma

Bancos de germoplasma são as estruturas físicas onde o material genético é conservado para a manutenção da variabilidade genética, visando a sua utilização imediata ou futura. Dessa forma, eles constituem a principal fonte de variabilidade genética e de possíveis características de interesse para os programas de melhoramento de uma espécie.

Para que a diversidade disponível nos bancos de germoplasma seja utilizada de forma adequada, é necessário que os acessos sejam

caracterizados, documentados e avaliados, de forma que o melhorista possa identificar os potencialmente úteis para os programas de melhoramento (Borém & Miranda 2005). Em termos gerais, a caracterização pode ser morfológica, reprodutiva, agronômica, bioquímica, citogenética e molecular, baseando-se na classificação dos acessos por seus caracteres qualitativos, enquanto a avaliação considera os caracteres quantitativos (Valois *et al*. 2001). Considerando que a caracterização morfológica depende da utilização de caracteres (descritores) estáveis, cuja identificação depende da avaliação dos acessos em diferentes locais e anos, a utilização de marcadores moleculares pode facilitar a etapa de caracterização dos bancos de germoplasma, já que não sofrem influência ambiental e podem ser avaliados em qualquer estágio do crescimento da planta. Dessa forma, o estudo da diversidade genética com marcadores moleculares, dentro dos bancos de germoplasma, gera informações que têm como objetivos otimizar a manutenção e o manejo das coleções e facilitar o acesso dos melhoristas à variabilidade disponível.

Um grande número de acessos de gramíneas e leguminosas com potencial forrageiro está disponível em diferentes bancos de germoplasma, sendo que seu uso ainda é incipiente. No banco de germoplasma do Centro Internacional de Agricultura Tropical (CIAT), estão armazenados 28158 acessos de espécies tropicais com potencial forrageiro, a maioria de leguminosas (Karia 2008). Na Embrapa Cerrados, cerca de 5000 acessos de leguminosas com potencial forrageiro foram introduzidos e avaliados, sendo que mais de 1500 pertencem ao gênero *Stylosanthes* Sw. (Karia & Andrade

1996). Em espécies como as do gênero *Stylosanthes* Sw., em que ainda não há uma lista de descritores disponíveis para caracterização morfológica, o uso de marcadores moleculares pode melhorar o conhecimento a respeito dos acessos armazenados nas coleções, incentivando seu uso em programas de melhoramento.

Pelas características destacadas no item anterior, os marcadores microssatélites vêm sendo muito utilizados para caracterização de bancos de germplasma, havendo uma série de trabalhos na literatura mostrando essa aplicação. Alguns desses trabalhos tentam relacionar a diversidade genética de acessos de acordo com a origem geográfica. Huang *et al*. (2002) estudaram a diversidade genética entre acessos de trigo coletados em 68 países, representando os cinco continentes e encontraram maior diversidade genética entre os acessos coletados no Oriente Médio em relação aos acessos de outras origens geográficas. Já em sorgo, foram estudados acessos da Ásia, Américas e diferentes regiões da África, sendo que a maior diversidade genética foi observada entre acessos do leste africano e a menor entre acessos da Ásia (Djè *et al*. 2000). Essas informações, além de auxiliarem no uso da diversidade genética, podem ser utilizadas para direcionar coletas para determinados locais quando se deseja aumentar a base genética da coleção.

Outros trabalhos estudam etnovariedades (*landraces*) e comparam sua diversidade à diversidade de variedades modernas. Asfaw *et al.* (2009) estudaram a diversidade genética de acessos representando etnovariedades de feijão comum coletados no leste da África através de marcadores microssatélites e compararam esses acessos à cultivares controle

representando os *pools* gênicos conhecidos de feijão (Andino e Mesoamericano). Foi possível observar que os acessos da Etiópia eram predominantemente mesoamericanos e os do Quênia predominantemente andinos e que houve pouca mistura entre esses *pools* gênicos. Além disso, foi observada uma grande diversidade genética entre os acessos, indicando que mais cuidados devem ser tomados para conservar as etnovariedades de feijão existentes nessa região da África. Essas informações também são importantes para o melhoramento de feijão dessa região, já que esses acessos podem ser fontes de características de interesse específicas para esses locais. Em trigo, microssatélites foram utilizados para estudar a diversidade genética de acessos representando etnovariedades e variedades modernas da China, revelando a presença de maior diversidade genética entre as etnovariedades e ressaltando a importância da sua conservação. Foi possível detectar que há diferença na diversidade genética entre os três genomas do trigo (A, B e D), mostrando que, na China, o genoma D sofreu maior pressão de seleção que os outros genomas. Ainda, esse trabalho sugere que, para a criação de uma coleção nuclear desses acessos, seria necessário incluir no mínimo 4% da coleção de base, para representar 70% da variabilidade genética existente (Hao *et al*. 2006). As coleções nucleares são definidas como o menor número possível de acessos, derivados de uma coleção de germoplasma que representam a maior parte da diversidade genética disponível na coleção e facilitam a utilização dos recursos genéticos.

Em feijão, Blair *et al*. (2009) utilizaram microsatélites para avaliar acessos de uma coleção nuclear e tentar identificar estrutura populacional e

associação com tamanho de semente. Foram identificados dois grupos dentro da coleção, relacionados com os dois *pools* gênicos de feijão e foram identificadas várias regiões associadas ao tamanho da semente, gerando informações importantes e que podem ajudar no direcionamento de cruzamentos dentro do programa de melhoramento de feijão. Em abacate, 16 microssatélites foram utilizados para avaliar 75 acessos de uma coleção na Espanha. No geral, houve uma diferenciação entre as raças de abacate existentes, mas, como muitos acessos da coleção eram híbridos inter-raciais, foi observada uma mistura de acessos de diferentes raças dentro de um mesmo grupo. Outra possível causa para essa mistura seria a classificação equivocada de alguns acessos. O conhecimento da relação das raças com a distribuição da diversidade genética na coleção de germoplasma de abacate pode auxiliar nos programas de melhoramento, já que as características morfológicas e agronômicas de cada raça são conhecidas (Alcaraz & Hormaza 2007).

Como será mostrado adiante, poucos trabalhos foram realizados a fim de estudar a diversidade genética e sua estrutura no gênero *Stylosanthes* Sw..

2.4 Sistema de Cruzamento em Plantas

O sistema de cruzamento é um dos fatores que mais influenciam a diversidade genética e a evolução dos genomas (Charlesworth & Wright 2001). Ele tem um papel crucial na composição genética das populações, visto que determina a freqüência dos genótipos individuais nas gerações, com grande

influência na distribuição e no conteúdo da variação genética dentro e entre populações (Brown 1990).

De acordo com o sistema reprodutivo, as plantas são geralmente divididas em três categorias: i) autógamas, ii) alógamas e iii) mistas. As espécies que apresentam até 5% de taxa de cruzamento são consideradas autógamas, enquanto as que apresentam mais de 95% de taxa de cruzamento são consideradas alógamas. As espécies que apresentam uma taxa intermediária de fecundação cruzada em relação à das espécies autógamas e alógamas são consideradas mistas.

As populações de espécies autógamas são constituídas por indivíduos altamente homozigóticos sendo que a heterozigose, originada pelos raros eventos de fecundação cruzada, tende a desaparecer com as sucessivas autofecundações (Borém & Miranda 2005). Dessa forma, essas populações tendem a ser homogêneas, concentrando a maior parte da variabilidade genética entre diferentes populações (Hamrick & Godt 1996). Em contrapartida, as populações de espécies alógamas são caracterizadas pela heterogeneidade, sendo cada indivíduo da população altamente heterozigótico e distinto dos demais (Borém & Miranda 2005). Em espécies alógamas, em geral, a maior parte de variabilidade genética se encontra dentro das populações (Hamrick & Godt 1996).

Os primeiros métodos para a avaliação do sistema de cruzamento de plantas baseavam-se na observação do comportamento de agentes polinizadores, no exame da morfologia floral e de resultados de experimentos controlados de polinização, gerando conclusões qualitativas a respeito do

sistema de cruzamento. Um avanço considerável constituiu na análise de progênies de plantas possuindo marcadores morfológicos conhecidos. Nessas análises, modelos estatísticos possibilitam quantificar as taxas de cruzamento das espécies (Clegg 1980, Shaw *et al*. 1981). A estimação da taxa de cruzamento através de marcadores morfológicos depende da escolha de caracteres estáveis e que não tenham influência na estimativa, o que torna esse processo trabalhoso e passível de erro (Miles 1983, 1985).

O surgimento dos marcadores moleculares possibilitou sua utilização na obtenção de estimativas mais precisas das taxas de cruzamento de diferentes espécies de plantas. Inicialmente, muitos trabalhos empregaram isoenzimas para avaliar a taxa de cruzamento em diferentes espécies como milho (Brown & Allard 1970), cevada (Brown *et al*. 1978), centeio (Vaquero *et al*. 1989) e *Arabidopsis thaliana* (L.) Heynh. (Abbott & Gomes 1989). Trabalhos com marcadores dominantes também vêm sendo realizados (Gaiotto *et al*. 1997, Beland *et al*. 2005) porém, após seu surgimento, os microssatélites vêm sendo os marcadores mais utilizados para estimar a taxa de cruzamento em plantas (Abdel-Ghani *et al.* 2004, O'Connell *et al*. 2004, Djè *et al*. 2004).

O conhecimento do sistema de cruzamento de uma espécie é fundamental em programas de melhoramento e conservação genética *ex situ* porque permite delinear estratégias que otimizem a amostragem da variabilidade genética durante as coletas, além de direcionar formas adequadas de multiplicação de sementes e a adoção de modelos mais adequados de melhoramento (Souza *et al*. 2003, Borém & Miranda 2005). Apesar de ser uma das características que mais influenciam os padrões de

variação genética em plantas (Hamrick & Godt 1996), informações sobre a taxa de cruzamento de muitos grupos ainda são limitadas.

Poucos estudos avaliaram o sistema de cruzamento em *Stylosanthes* Sw. e, apesar de suas espécies serem consideradas autógamas (Bray & Hutton 1976), esses trabalhos mostraram a ocorrência de cruzamentos. Stace (1982) estudou um loco de isoenzima e observou aproximadamente 2% de taxa de cruzamento em *S. scabra* Vog.. Em *S. capitata* Vog.*,* Miles (1983) observou a presença ou ausência de pilosidade nas estípulas e obteve uma estimativa média de 20% de fecundação cruzada, enquanto que, em *S. guianensis* (Aubl.) Sw., Miles (1985) estudou a cor da flor e observou 14% de taxa de cruzamento. Porém, como a taxa de cruzamento em *Stylosanthes* Sw. depende da presença e atividade de insetos para polinização, o caráter cor da flor pode ter causado um viés na estimativa da taxa de cruzamento em *S. guianensis* (Aubl) Sw., já que pode ter uma influência na ação dos polinizadores (Miles 1985).

2.5 Marcadores Moleculares em *Stylosanthes* Sw.

A taxonomia de *Stylosanthes* Sw. com base em marcadores morfológicos é confusa, havendo uma série de controvérsias em relação às espécies e variedades consideradas válidas (Maass & Sawkins, 2004). Trabalhos utilizando diferentes técnicas moleculares têm tentado auxiliar na elucidação das relações dentro do gênero.

Kazan *et al*. (1993) utilizaram marcadores RAPD a fim de elucidar as relações dentro da espécie *S. guianensis* (Aubl.) Sw., uma das mais

controversas dentro do gênero. Seu trabalho confirmou a classificação proposta por Ferreira & Costa (1979), que trata as diferentes variedades propostas por t' Manetje (1984) como diferentes espécies, já que a divergência genética entre essas variedades foi da mesma proporção da observada entre diferentes espécies. Vieira *et al*. (1997) também estudaram as relações taxonômicas em *Stylosanthes* Sw. com foco no complexo de *S. guianensis* (Aubl.) Sw. através de marcadores RAPD e, novamente, os dados corroboraram com a classificação proposta por Ferreira & Costa (1979), por observarem uma grande distância genética entre as variedades propostas por 't Manetje (1984). Também na tentativa de elucidar as relações das espécies no grupo *S. guianensis* (Aubl.) Sw., Vander Stappen *et al*. (1999a) seqüenciaram a região de DNA ribossomal ITS1 (*Internal Transcribed Spacer*) e também verificaram ampla variação entre as variedades botânicas de 't Manetje (1984) .

As relações entre as espécies do gênero foram estudadas com base na variação de regiões de cloroplasto e o gênero foi dividido em quatro grupos, i) formado pelo complexo *S. guianensis* (Aubl.) Sw. e algumas espécies relacionadas, ii) formado por *S. híspida* Michaux, acessos tetraplóides de *S. hamata* (L.) Taub.*, S. sympodialis* Taub.*, S. humilis* Kunth*, S. leiocarpa* Vog.*, S. angustifólia* Vog. e alguns acessos de *S. scabra* Vog., iii) formado por *S. calcicola* Small*, S. viscosa* Sw., acessos diplóides de *S. hamata* (L.) Taub. e *S. fruticosa* (Retz.) Alston, além de alguns acessos de *S. scabra* Vog.*, S. capitata* Vog. e um acesso de *S. grandifolia* Ferr. et Costa e iv) formado por *S. macrocephala* Ferr. et Costa e outros acessos de *S. capitata* Vog. não incluídos

no terceiro grupo. No geral, essas relações concordaram com a classificação taxonômica de Ferreira & Costa (1979). Esse trabalho também identificou o doador materno de algumas espécies tetraplóides, *S. humilis* Kunth foi identificado como parental materno de *S. sympodialis* Taub. e de *S. hamata* (L.) Taub. tetraplóide, enquanto *S. viscosa* Sw. foi identificado como parental materno de *S. scabra* Vog. e *S. macrocephala* Ferr. et Costa foi identificado como parental materno de *S. capitata* Vog. (Gillies & Abbott, 1996).

Marcadores STS (Sequence-Tagged Sites) foram utilizados de forma isolada e em conjunto com RFLP's para estudar as relações interespecíficas em *Stylosanthes* Sw.. Esses marcadores se baseiam na utilização de *primers* complementares à regiões de DNA de uma ou poucas cópias no genoma para amplificação via PCR e busca por polimorfismos.

Vander Stappen *et al*. (1999b) utilizaram 19 marcadores STS presentes em nove genes de *Stylosanthes* Sw., em sua maioria de cloroplasto, para estudar a variação intra e inter-específica em 63 genótipos, representando 24 espécies do gênero. O gênero foi subdivido em três grupos que, em geral, concordam com os grupos propostos com base em regiões de cloroplasto por Gillies & Abbott (1996) e também com a taxonomia já descrita.

Liu *et al.* (1999) utilizaram STS e RFLP's para estudar 6 acessos não classificados e 24 espécies conhecidas de *Stylosanthes* Sw.. Com base nesses dados, eles classificaram essas espécies em nove genomas basais, i) *S. seabrana* Maass. et t' Manetje/*S. hamata* (L.) Taub. (A), ii) *S. viscosa* Sw. (B), iii) *S. humilis* Kunth (C), iv) *S. macrocephala* Ferr. et Costa /*S. bracteata* Vog. (D), v) *S. pilosa* Ferr. et Costa (E), vi) *S. leiocarpa* Vog. (F), vii)*S. guianensis*

(Aubl.) Sw. (G), viii) *S. tomentosa* Ferr. et Costa (H) e ix) *S. calcicola* Small (I). Os acessos poliplóides foram classificados em cinco grupos, baseados nas suas prováveis estruturas genômicas. As espécies *S. scabra* Vog., *S.* aff. *scabra*, *S. sericeiceps* Blake, *S.* aff. *hamata* e *S. tuberculata* Blake apresentaram uma estrutura genômica AABB. Uma estrutura AACC foi encontrada para *S. mexicana*, *S. subsericea* Blake e *S. sundaica* Taub., enquanto *S. capitata* Vog. apresentou uma estrutura DDEE, *S. sympodialis* Taub. apresentou uma estrutura AAFF e *S. erecta* Welw ex Baker apresentou uma estrutura AABBXX, sendo XX um genoma desconhecido.

As regiões ITS1 e ITS2 também foram utilizadas para inferir relações filogenéticas no gênero *Stylosanthes* Sw.. Vander Stappen *et al*. (2002) utilizaram a comparação das seqüências dessas regiões em 119 indivíduos, representando 36 espécies de *Stylosanthes* Sw. e sete espécies de grupos relacionados. Foi observada variação intraespecífica nessas regiões tanto em diplóides como em poliplóides e os grupos formados concordaram com os dados de regiões de cloroplasto e, em parte, com a classificação taxonômica clássica de (t' Manetje 1984).

Marcadores moleculares foram também aplicados para estudar a diversidade genética de algumas espécies. Liu (1997) avaliou 100 acessos de *S. scabra* Vog. de um banco de germoplasma da Austrália, representando a distribuição geográfica dessa espécie, utilizando 28 *primers* de RAPD. Foi detectada uma baixa distância genética entre esses acessos (0,059) sendo que, aqueles de origem brasileira apresentaram uma menor diversidade do que os coletados na Colômbia e Venezuela. Esses acessos foram divididos em cinco

grupos sendo que não houve correlação entre esses grupos e os grupos observados através de avaliações morfoagronômicas.

A diversidade genética de *S. humilis* Kunth coletados no México foi estudada com marcadores AFLP e comparada à diversidade de acessos coletados na América do Sul (Vander Stappen *et al.* 2000). Os marcadores foram capazes de separar os acessos de ambas as regiões, concordando com dados prévios que indicavam a existência desses dois *pools* gênicos distintos. Os padrões geográficos da variabilidade genética de 111 acessos de *S. humilis* Kunth e *S. viscosa* (L.) Sw. foram investigados utilizando marcadores AFLP (Sawkins *et al*. 2001). A similaridade genética entre os acessos foi alta nessas espécies (0,72 em *S. humilis* Kunth e 0,67 em *S. viscosa* (L.) Sw.) e, no geral, os grupos formados estavam de acordo com a distribuição geográfica.

Estudos utilizando marcadores moleculares também foram realizados para avaliar a diversidade genética em acessos de bancos de germoplasma brasileiros. A variabilidade genética de 87 acessos da Embrapa Cerrados, pertencentes à espécie *S. macrocephala* Ferr. et Costa foi avaliada através de 161 marcadores RAPD (Barros *et al*. 2005), sendo observada uma alta diversidade genética entre acessos dos estados da Bahia e de Minas Gerais, além de uma tendência de separação por bacias hidrográficas. Karia (2008) estudou a diversidade genética de acessos da Embrapa Cerrados, pertencentes à espécie *S. guianensis* (Aubl.) Sw., utilizando sete marcadores microssatélites desenvolvidos por Vander Stappen *et al*. (1999c). Foi observada uma tendência dos acessos a se agruparem de acordo com as variedades botânicas

descritas por Ferreira & Costa (1979), sendo que a correlação entre os dados moleculares e morfoagronômicos foi significativa, porém de baixa magnitude.

A relação de dados moleculares com a resistência à antracnose e com a resistência à seca também foi avaliada em algumas espécies de *Stylosanthes* Sw.. Chang-Shun *et al*. (2004) estudaram acessos de *S. guianensis* (Aubl.) Sw. resistentes e suscetíveis à antracnose através de AFLP's e não detectaram uma relação entre os agrupamentos formados e a resistência à essa doença. Marcadores RAPD foram utilizados para desenvolver um mapa em uma F_2 de *S. scabra*, visando a detecção de QTL's associados a caracteres associados à resistência à seca (Thumma *et al*. 2001). Vários QTL's puderam ser identificados relacionados aos caracteres eficiência de transpiração (ET), área foliar (AF) e discriminação de isótopos de carbono (DC), sendo que os QTL's para ET e DC estavam localizados nos grupos de ligação 5 e 11, enquanto os QTL's para AF estavam localizados nos grupos 13 e 24.

Um mapa genético parcial também foi desenvolvido para uma F_2, proveniente de um cruzamento entre *S. scabra* Vog. cv Seca e *S. fruticosa* (Retz.) Alston , através de 81 marcadores RAPD. Foram identificados sete grupos de ligação, com um comprimento de 630,4 CM e com intervalo médio de 39,4 cM entre os marcadores. Esse mapa parcial foi construído para facilitar a ancoragem de mais marcadores RAPD e STS, visando à localização de QTL's relacionados à resistência à antracnose (Chandra 2006).

Os dados apresentados acima mostram diferentes aplicações de marcadores moleculares em *Stylosanthes* Sw., desde seu uso para elucidar relações taxonômicas dentro do gênero, quanto para auxiliar no conhecimento

da diversidade genética de espécies e identificar regiões relacionadas a caracteres de interesse. Esses métodos, juntamente com dados morfoagronômicos e o conhecimento de características ecológicas dos locais de coletas dos acessos constituem uma ferramenta poderosa para auxiliar na manutenção e caracterização de bancos de germoplasma, na organização de recursos genéticos, na identificação de acessos duplicados, no estudo de estrutura populacional, no planejamento de novas coletas e na construção de coleções menores (Zong *et al*. 2009, Westman & Kresovich, 1997). A disponibilidade dessas informações pode auxiliar e acelerar os programas de melhoramento de *Stylosanthes* Sw., aumentando a eficiência da utilização dos bancos de germoplasma desse gênero e auxiliando no planejamento de novas coletas.

3 Objetivos

Geral: Estimar a taxa de cuzamento e acessar a diversidade genética em espécies de *Stylosanthes* Sw. através de marcadores moleculares microssatélites.

Específicos

- ✓ Isolar e seqüenciar locos do genoma de *S. capitata* Vog., *S. guianensis* (Aubl.) Sw. e *S. macrocephala* Ferr. et Costa que contenham microssatélites,

- ✓ Desenhar *primers* específicos para amplificar os locos de microssatélites isolados e selecionados,

- ✓ Caracterizar cada loco de microssatélite isolado com relação ao tipo de repetição, número e tamanho dos alelos amplificados, temperatura de anelamento, heterozigosidade observada e esperada em materiais divergentes de *Stylosanthes* Sw.,

- ✓ Utilizar os microssatélites desenvolvidos para acessar a diversidade genética e estimar a taxa de cruzamento em *S. capitata* Vog., *S. guianensis* (Aubl.) Sw. e *S. macrocephala* Ferr. et Costa.

4 Artigo I

"Isolation and characterization of microsatellite loci in tropical forage *Stylosanthes capitata* Vogel"

Publicado na Revista Molecular Ecology Resources (9, 192-194, 2009)

Autores: Melissa de O. Santos, Rodrigo P. Sassaki, Lucimara Chiari, Rosângela M. S. Resende, Anete P. Souza.

Molecular Ecology Resources (2009) 9, 192–194 doi: 10.1111/j.1755-0998.2008.02308.x

PERMANENT GENETIC RESOURCES

Isolation and characterization of microsatellite loci in tropical forage *Stylosanthes capitata* Vogel

M. O. SANTOS,* R. P. SASSAKI,* L. CHIARI,† R. M. S. RESENDE† and A. P. DE SOUZA*
*Centro de Biologia Molecular e Engenharia Genética, Departamento de Genética e Evolução, Universidade Estadual de Campinas, CP 6010, Barão Geraldo, Campinas, SP, Brazil, CEP 13083-970, †Empresa Brasileira de Pesquisa Agropecuária (Embrapa) – Gado de Corte, BR 262, Km 4, Campo Grande, MS, Brazil, CEP 79002-970

Abstract

***Stylosanthes capitata* is an important tropical pasture legume. Knowledge of genetic diversity and structure of *S. capitata* populations is of great importance for the conservation and germplasm management of this species. Thus, eight microsatellite markers were developed from an *S. capitata*-enriched library. They were characterized in 20 accessions from the germplasm collection of the Empresa Brasileira de Pesquisa Agropecuária (Embrapa). The observed and expected heterozygosities ranged from 0.16 to 0.85 and from 0.40 to 0.85, respectively. These microsatellites are the first set of molecular markers from this species and will contribute towards studies of genetic diversity, conservation and breeding of *S. capitata*.**

Keywords: Fabaceae, microsatellites, *Stylosanthes*

Received 24 April 2008; revision accepted 9 June 2008

The genus *Stylosanthes* Sw. (Fabaceae) comprises about 40 species, distributed in the tropical areas of America, Africa and Southeast Asia (Costa & Ferreira 1984; Mannetje 1984; Williams *et al*. 1984). The major centre of origin and diversity of *Stylosanthes* is in Brazil, where 25 out of the 40 species of this genus are found (Karia & Andrade 2000). The *Stylosanthes* plants have a high protein content, good development in relatively poor soil, easy propagation and can incorporate nitrogen to the soil because of their symbiosis with the bacteria of the *Rhizobium* genus (Baldión *et al*. 1975). Because of these *Stylosanthes* is used together with the *Brachiaria* species in the formation of pastures.

Stylosanthes capitata belongs to the Styposanthes Section and is considered an allotetraploid which comes from the hybridization of *Stylosanthes macrocephala* and *Stylosanthes pilosa* (Liu *et al*. 1999). Despite the importance of the genus and the Brazilian origin of most of its species, no molecular analysis has been carried out to discover the diversity of the Brazilian accessions. The isolation and characterization of novel microsatellites from a genomic library of *S. capitata*, which are potentially useful in the future genetic studies of this species, are reported here.

Correspondence: Anete Pereira de Souza, Fax: 55-19-3521-1089; E-mail: anete@unicamp.br

Genomic DNA was isolated from leaves using the (cetyltrimethyl ammonium bromide) CTAB method (Doyle & Doyle 1990) with modifications made for the genus by Bonato *et al*. (2002). A genomic-enriched library was constructed following the protocol described by Billotte *et al*. (1999). The genomic DNA was digested with RSA*I* and enriched in $(CT)_8$ and $(GT)_8$ repeats. Enriched fragments were amplified by polymerase chain reaction (PCR), ligated into a pGEM T-Easy vector (Promega) and then transformed into competent XL1-blue *Escherichia coli* cells. The positive clones were selected using the β-galactosidase gene and then grown overnight in an HM/FM medium with ampicillin. After PCR, 48 positive clones were sequenced in both directions using the T7 and SP6 primers as well as the BigDye version 3.1 terminator kit (Perkin Elmer, Applied Biosystems) with an ABI PRISM 377. The sequences were assembled and edited in Seqman (DNAStar). The repetitive regions were found using the Simple Sequence Repeat Identification Tool (Temnykh *et al*. 2001). Nineteen of these clones contained microsatellite sequences with more than five repeats and adequate flanking regions for primer design. The software Primer Select (DNAStar) was used to design 19 primer pairs flanking the repetitive regions.

The test and characterization of polymorphism of the isolated microsatellite loci was performed using 20 accessions

Table 1 Characteristics of eight microsatellite loci from *Stylosanthes capitata*. Shown are the loci names, the GenBank Accession number, the forward (F) and reverse (R) primer sequence, repeat motif, number of alleles (N), product size range in base pairs, observed (H_O) and expected (H_E) heterozygosities, P value HWE. *departs significantly from HWE at $P < 0.05$ after Bonferroni correction, ** evidence of null alleles detected at $P < 0.05$

Locus	GenBank Accession no.	Primer sequences (5′–3′)	Repeat motif	N	Size range (bp)	H_O	H_E	P value HWE
SC 18-01 B3	EU337004	F: 5′-TGGAGACAACACCCTTATG-3′ R: 5′-ATTCTATTACTCTTGCCTTTTCT-3′	$(GA)_5$	2	222–225	0.60	0.42	0.055
SC 18-01 C7B	EU337005	F: 5′-CTTCTTTATCCCCACCTTTTT-3′ R: 5′-AGCACACTCTTTGATGATGAG-3′	$(TC)_{17}$	5	318–330	0.16	0.72	0.000*,**
SC 18-01 E11	EU337006	F: 5′-GGCTAAAGAACGGCTAATG-3′ R: 5′-TCGAAAGATCCAAGAACAAA-3′	$(GT)_7$	2	235–239	0.55	0.40	0.090
SC 18-01 G4B	EU337007	F: 5′-GCATAGCAGCATAGGTAGTAAA-3′ R: 5′-ATGCCAGGGCTGATAGAAG-3′	$(TG)_3(CA)_2$	2	250–255	0.75	0.47	0.007
SC 18-01 H6A	EU337008	F: 5′-ATTCTCAATTTCACTTCTTCAA-3′ R: 5′-CCAACCCTTTCTTTTCAAT-3′	$(TG)_{10}(AG)_{12}$	7	255–280	0.85	0.85	0.032
SC 18-01T F11A	EU337009	F: 5′-CCGACCAAGGGGGATGTC-3′ R: 5′-AAGTAGCAGCGGCGAGACC-3′	$(AG)_6$	2	188–186	0.85	0.49	0.001*
SC 18-01T F6A	EU337010	F: 5′-AACACGAGGATGAAACGAA-3′ R: 5′-AACCCTATCACACTAAACTCACA-3′	$(TC)_8$	2	204–206	0.40	0.38	0.765
SC 18-02T G11	EU337011	F: 5′-ATGAGAAATGAAAGATAATGGA-3′ R: 5′-ATACCGAGGAGGATAAACAAG-3′	$(GA)_3(TGG)_2$	2	285–302	0.85	0.49	0.001*

Table 2 Cross–species amplification of eight microsatellite loci from *Stylosanthes capitata* in *Stylosanthes guianensis* and *Stylosanthes macrocephala*. Successful amplification (+) and failed amplification (–) are indicated

Species	SC 18-01 B3	SC 18-01 C7B	SC 18-01 E11	SC 18-01 G4B	SC 18-01 H6A	SC 18-01T F11A	SC 18-01T F6A	SC 18-02T G11
S. guianensis	–	+	–	+	–	+	+	–
S. macrocephala	–	+	–	+	+	–	+	+

of the germplasm collection of this species, which is located in the Empresa Brasileira de Pesquisa Agropecuária (Embrapa) Gado de Corte.

PCR amplifications were performed in a 20 μL reaction volume using PTC-200 (MJ Research). Final concentrations for optimizing reactions were 1× PCR buffer, 1.5 mM $MgCl_2$, 0.3 mM of each dNTP (Invitrogen), 0.8 μM of each primer, 1 U de *Taq* DNA polymerase (Invitrogen) and 20 ng of genomic DNA. The amplification protocol consisted of an initial denaturation at 94 °C for 1 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, 72 °C elongation for 1 min and a final extension at 72° for 5 min. The amplification products were separated in 6% denaturing polyacrylamide gels and visualized by silver–staining as described by Creste *et al.* (2001). The allele scoring was done using the 10-bp ladder (Invitrogen), and to ensure that the alleles were correctly scored, a proportion of individuals were genotyped a second time.

Out of the 19 primer pairs designed, eight were polymorphic, with the number of alleles per locus ranging from two to seven, with an average of three alleles per locus (Table 1). Descriptive statistics and the test for Hardy–Weinberg equilibrium (HWE) were performed using Tools for Genetic Population Analysis (TFPGA; Miller 1997). The test for the evidence of null alleles was performed using MICRO-CHECKER 2.2.3 (Van Oosterhout *et al.* 2004). The observed and expected heterozygosities ranged from 0.16 to 0.85 (0.62 on average) and from 0.40 to 0.85 (0.53 on average), respectively, for these eight loci. Three loci departed significantly from HWE ($P < 0.05$) after Bonferroni correction, and for the locus SC 18-01 C7B, this departure could be probably explained by the evidence of null alleles. The linkage disequilibrium was tested using PopGene 1.32 (Yeh *et al.* 1998) and no disequilibrium was detected among all loci. The cross-species amplification was tested on *S. macrocephala* and *Stylosanthes guianensis* (Table 2).

These microsatellites are the first set of molecular markers from this species, and they will be used to study the genetic diversity of the Brazilian germplasm collections and the genetic structure of natural populations of *S. capitata*.

Acknowledgements

The authors would like to thank Maria Imaculada Zucchi for the support during the analysis of the data; Fundação de Amparo à Pesquisa do Estado de São Paulo for the financial support during

the development of this research (2005/51010-0). Both M.O. Santos and R.P. Sassaki received DR (2005/52211-9) and IC (2006/52845-0) grants from FAPESP. A.P. Souza is recipient of a research fellowship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

References

Baldión R, Lozano JC, Grof B (1975) Evaluación de la resistência de *Stylosanthes* spp. a la antracnosis (*Colletotrichum gloeosporioides*). *Fitopatologia*, **10**, 104–108.

Billotte N, Lagoda PJR, Risterucci AM, Baurens FC (1999) Microsatellite-enriched libraries: applied methodology for the development of SSR markers in tropical crops. *Fruits*, **54**, 277–288.

Bonato ALV, Verzignassi JR, Resende RMS, Fernandes CD, Leguizamón GOC (2002) Extração de DNA genômico de *Stylosanthes* spp. Comunicado Técnico 78. Empresa Brasileira de Pesquisa Agropecuária (Embrapa), Campo Grande, Mato Grosso do Sul, Brazil.

Costa NMS, Ferreira MB (1984) Some Brazilian species of *Stylosanthes*. In: *The Biology and Agronomy of Stylosanthes* (eds Stacce HM, Edye LA), pp. 23–48. Academic Press, Sydney, Australia.

Creste S, Tulmann Neto A, Figueira A (2001) Detection of single sequence repeat polymorphisms in denaturing polyacrylamide sequencing gels by silver staining. *Plant Molecular Biological Reporter*, **19**, 299–306.

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. *Focus*, **12**, 13–15.

Karia CT, Andrade RP (2000) Uso de *Stylosanthes* em pastagens no Brasil. In: *Anais do Simpósio de Forragicultura e. Pastagens: Temas em evidência*, pp. 471–475. Universidade Federal de Lavras, Lavras, Minas Gerais, Brazil.

Liu CJ, Musial JM, Thomas BD (1999) Genetic relationship among *Stylosanthes* species revealed by RFLP and STS analyses. *Theoretical and Applied Genetics*, **99**, 1179–1186.

Mannetje L (1984) Considerations on the taxonomy of the genus *Stylosanthes*. In: *The Biology and Agronomy of Stylosanthes* (eds Stace HM, Edye LA), pp. 1–21. Academic Press, Sydney, Australia.

Miller MP (1997) Tools for population genetic analysis (TFPGA) 1.3. A Windows program for the analysis of allozyme and molecular population genetic data. Computer software distributed by the author, www.marksgeneticsoftware.net.

Temnykh S, DeClerck G, Lukashova A, Lipovich L, Catinhour S, McCouch S (2001) Computational and experimental analysis of microsatellites in rice (*Oryza sativa* L.): frequency, length variation, transposon associations, and genetic marker potential. *Genome*, **11**, 1441–1452.

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

Williams RJ, Reid R, Shultze- Kraft R, Costa NMS, Thomas BD (1984) Natural distribution of *Stylosanthes*. In: *The Biology and Agronomy of* Stylosanthes (eds Stacce HM, Edye LA), pp. 73–101. Academic Press, Sydney, Australia.

Yeh FC, Rong-Cai Y, Boyle T (1998) *PopGene*, Version 1.31. Center for International Forestry Research, University of Alberta, Edmonton, Alberta, Canada.

5 Artigo II

"Tropical Forage Legumes: I Estimation of the Outcrossing Rate of Important *Stylosanthes* species"

A ser submetido à revista Theoretical and Applied Genetics

Autores: Melissa de O. Santos-Garcia, Rosângela M. S. Resende, Lucimara Chiari, Maria I. Zucchi, Anete P. Souza.

Tropical Forage Legumes: I Estimation of the Outcrossing Rate of Important *Stylosanthes* Species

M O Santos-Garcia[1], R M S Resende[2], L Chiari[2], M I Zucchi[3], A P Souza[1,4]

[1] Centro de Biologia Molecular e Engenharia Genética, Universidade Estadual de Campinas, Barão Geraldo, Campinas, SP, Brazil. CP 6010, CEP 13083-970.

[2] Empresa Brasileira de Pesquisa Agropecuária (Embrapa) - Gado de Corte. BR 262, Km 4. Campo Grande, MS, Brazil. CEP 79002-970.

[3] Pólo Apta Centro Sul – Rod. SP 127 km 30, Piracicaba – SP, Brazil, CP 28, CEP 13400-970.

[4] Departamento de Biologia Vegetal, Instituto de Biologia, Universidade Estadual de Campinas, CP 6109, Campinas 13083-970, SP, Brazil.

Keywords: microsatellite enriched library, autogamy, mixed mating system.

*Corresponding author: Anete Pereira de Souza. Centro de Biologia Molecular e Engenharia Genética. Universidade Estadual de Campinas, Campinas, SP, Brazil. CP 6010. CEP 13083-970.

E-mail: anete@unicamp.br

Phone: 55-19-3521-1132

FAX: 55-19-3521-1089

Abstract

Stylosanthes capitata and *S. guianensis* are important forage legumes for tropical areas. Estimates of outcrossing rate are extremely important to assist breeders in choosing the most suitable breeding methods. The only available estimates of *S. capitata* and *S. guianensis* outcrossing rates were based on morphological markers. Here we describe the estimation of the outcrossing rate for *S. capitata* and *S. guianensis* using microsatellite markers. We also describe the development of a new set of microsatellite loci for *S. capitata.* A total of 49 new microsatellites were characterized in 20 *S. capitata* accessions from the germplasm collection of the Brazilian Agricultural Research Corporation (Embrapa) Beef Cattle. Of the 49 microsatellites, 14 were polymorphic with an average of 3.3 alelles per locus and observed and expected heterozygosities means of 0.33 and 0.49, respectively. The outcrossing rates were estimated in *S. capitata* and *S. guianensis* populations of 20 progenies that consisted of ten individuals each. The multilocus outcrossing rate for *S.capitata* was estimated using 10 polymorphic loci, while five microsatellites were used for *S. guianensis.* The outcrossing rates for *S. capitata* and *S. guianensis* were 31% and 26% respectively, suggesting a mixed mating system with predominance of autogamy. The data on outcrossing rate described here are potentially useful for breeding programs and for maintenance of germplasm collections o these *Stylosanthes* species.

Introduction

The genus *Stylosanthes* Sw. belongs to the tribe Aeschynomeneae, subtribe Stylosanthinae, Fabaceae family and includes 48 herbaceous-subshrub species (Costa 2006). Most *Stylosanthes* species are distributed in tropical, subtropical and warm temperate areas of Americas. The major diversity center of this genus is Central Brazil (Ferreira and Costa 1979, Stace and Cameron 1984), which includes 45% of all *Stylosanthes* species, as well as the greatest phenotypic variation and endemism (Costa 2006). The other diversity center corresponds to the region of Mexico and Caribbean Islands, in Central America (Stace and Cameron 1984). *Stylosanthes* species are among the most important sources of pasture legumes for tropical and subtropical regions (Edye and Cameron 1984), being also used for soil improvement through nitrogen fixation and for recovery of degraded wastelands (Pathak et al. 2004, Kelemu et al. 2005, Chandra et al. 2006).

The species *Stylosanthes capitata*, in the Styposanthes Section, is an allotetraploid that most likely was derived from hybridization between *S. macrocephala* and *S. pilosa* (Gillies and Abbott 1996, Liu et al. 1999). In South America, *S. capitata* has been considered promising for soils with low pH and high saturation of aluminium and manganese (Grof et al. 1979). Besides, it has shown positive effects on animal production (Thomas et al. 1987). *S. guianensis*, Stylosanthes section, is a diploid species (Stace and Cameron 1984) with great morphological diversity, being the most widespread and one of the most promising among *Stylosanthes* species. It produces great amounts

of dry matter and retains leaves during dry season (Williams et al. 1984, Maass and Sawkins 2004). *S. guianensis* is native to South and Central America, where it is widely distributed except for the equatorial zone (Williams et al. 1984). A more widespread exploitation of *Stylosanthes* species has been limited by their susceptibility to anthracnose, caused by *Colletotrichum gloeosporioides*, poor persistence of several varieties and low seed yield (Phaikaew et al. 2004). *Stylosanthes* breeding programs aim to overcome those constraints as well as to increase dry matter (Cameron et al.1984, Cameron et al. 1997, Chandra et al. 2006).

The genus *Stylosanthes* is considered to be mainly self-pollinated (Bray and Hutton, 1976) and seed production during germplasm management has been performed according to this assumption (Cameron et al. 1984). Contrastingly, few reports show occurrence of outcrossing in some of the species using an isozyme locus (Stace 1982) and morphological markers (Miles 1983, 1985). Data on outcrossing rates are relevant in germplasm acquisition, preservation of the genetic integrity of an accession or released cultivar and for stabilishing efficient plant breeding programs (Maass and Torres 1998).

Microsatellites or simple sequence repeats (SSRs) have proven particularly useful for estimating mating systems (Karasawa et al. 2007, Azevedo et al. 2007, O'Conell et al. 2004) since they are codominant, highly polymorphic and widely distributed over the genome. Few microsatellites are available in literature for *S. capitata* (Santos et al, 2009a) and the development of a higher number of microsatellites is of great importance for future studies in this species. Considering that the knowledge on *S. capitata*

and *S. guianensis* mating systems current available was based on morphological markers, here we report the development of new microsatellite loci for *S.capitata* and the estimation of the outcrossing rate in *S. capitata* and *S. guianensis* using microsatellite markers.

Materials and Methods

Development of *S. capitata* microsatellite loci

Samples of genomic DNA were extracted from fresh leaves of a single plant of *S. capitata* using a CTAB method (Doyle and Doyle 1990) with modifications made for the genus by Bonato et al. (2002). A genomic-enriched library was constructed according to Billotte et al. (1999). The genomic DNA was digested with RSA *I* and enriched using $(CT)_8$ and $(GT)_8$ probes. Enriched fragments were amplified by polymerase chain reaction (PCR), ligated into a pGem- T Easy vector (Promega) and then transformed into competent XL1-Blue *Escherichia coli* cells. Positive clones were selected using the β—galactosidase gene and grown overnight on an HM/FM medium with ampicilin. After PCR amplification, 96 positive clones were sequenced in both directions in an ABI PRISM® 377, using the T7 and SP6 universal primers and the v3.1 Big Dye terminator kit (PerkinElmer Applied Biosystems). Sequences were assembled and edited in Seqman (DNAStar).

Repetitive DNA regions were identified using the Simple Sequence Repeat Identification Tool (Temnykh et al. 2001). Clones containing microsatellite with dinucleotide motifs were selected if more than five repeats

were present. Those clones containing trinucleotide motifs were selected if they had more than four repeats and the ones containing tetranucleotide, pentanucleotide and hexanucleotide motifs were selected if there were more than three repeats. Compound microsatellites were considered if more than five repeats in total were present and they were not separated by more than three base pairs. Clones with flanking regions suitable for primer design were used to design primer pairs with the software Primer Select (DNAStar). Primers, typically consisting of 20-22 nucleotides were designed within the melting temperature range of 45 and 65°C, a minimum product length of 150 base pairs, a maximum product length of 300 bp and a maximum of 4°C difference between the temperatures of forward and reverse primers.

The test of polymorphism and characterization of the isolated microsatellite loci were performed using 20 accessions of the germplasm collection of Brazilian Agricultural Research Corporation (Embrapa) Beef Cattle (Table 1). Cross-transferability was tested in *S. guianensis* and *S. macrocephala*.

DNA sample of one individual plant from each of the 20 accessions were extracted using the CTAB method described above. PCR amplifications were performed in a 20 µl reaction volume using a PTC-200 (MJ Research) PCR thermocycler. Final concentrations of optimized reactions mixtures consisted of 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (Invitrogen), 0.8 µM of each primer, 1U de *Taq* DNA polymerase (Invitrogen) and 20ng of genomic DNA. Initially, all 52 primer pairs were submitted to an amplification program in which the primer annealing temperature was 60ºC. The primers that did not

show a satisfactory DNA amplification at 60ºC were submitted to a gradient program to determine the annealing temperature or to a touchdown program (Don et al. 1991). The amplification protocol consisted of an initial denaturation at 94ºC for 1 min, 30 cycles of denaturation at 94ºC for 1 min, annealing at a specific temperature for 1 min and 72ºC elongation for 1 min, and then a final extension at 72º for 5 min. PCR amplification products were evaluated on agarose gels stained with ethidium bomide, under UV radiation. The suitable DNA fragments were separated in 6% denaturing polyacrylamide gels and visualized by silver-staining as described by Creste et al. (2001). The allele scoring was done in comparison to a 10pb DNA ladder (Invitrogen).

Descriptive statistics were performed using Tools for Population Genetic Analysis (TFPGA) (Miller 1997) and the linkage disequilibrium was tested using Popgene 1.32 (Yeh and Boyle 1997).

Table 1 – List of the 20 accessions of *S. capitata* belonging to the Embrapa Beef Cattle germplasm collection, which were used for characterization of the developed microsatellite loci and as maternal parents in mating system determination. The respective number at Embrapa Beef Cattle germplasm collection (CNPGC), the number in CIAT collection and place of origin are listed.

Sample code	CNPGC	CIAT	Sample code	CNPGC	CIAT
1	1019	n.a	11	2190	n.a
2	1049	n.a	12	2196	n.a
3	1058	n.a	13	2216	n.a
4	1059	n.a	14	96	n.a
5	1064	n.a	15	1084	1914
6	1071	n.a	16	1165	1504
7	1326	n.a	17	1178	11096
8	141	n.a	18	1179	11097
9	2139	n.a	19	1182	11280
10	2188	n.a	20	1469	11096

*n.a – not available. [1]The place of origin refers to Brazilian States and Venezuela.

Mating System determination

Thirty accessions of *S. capitata* and 30 accessions of *S. guianensis* from the Germplasm Collection of Embrapa Beef Cattle were grown in the field in two randomized block design experiments under natural conditions in order to obtain maternal progenies. From those thirty accessions, 20 were randomly chosen for the progeny array of each species. In *S. capitata*, these 20 individuals belong to the same accessions used for characterization of the new microsatellites (Table 1) while, in *S. guianensis*, these accessions were used for the identification of polymorphic loci (Table 2). Pollinated seeds from the 20 maternal plants were grown in the year of 2005 and 10 plants per progeny were evaluated, totalizing 200 individuals for each species.

Table 2 – *S. guianensis* accessions selected as maternal plants for the progeny array. Showed are the accession numbers in the Embrapa collection, the origin of the accession when available and additional information about some of the accessions.

Sample code	Accession Number CNPGC	Sample code	Accession Number CNPGC
1	1461	11	2003
2	1594	12	1527
3	1512	13	1519
4	1557	14	1514
5	2023	15	1468
6	1515	16	1601
7	1477	17	1544
8	1677	18	1678
9	1528	19	1564
10	1561	20	1573

Genomic DNA samples were isolated from fresh leaves using the CTAB method as described before. For *S. capitata,* ten polymorphic microsatellite loci were chosen for analysis of the mating system, based on the expected heterozygosity value and on the quality of the DNA products for genotyping.

For *S. guianensis*, five polymorphic microsatellites were identified among the 38 loci available in the literature for this species (Vander Stappen et al. 1999b, Santos et al. 2009b) and used for DNA amplification of the entire population. DNA amplification was performed using the optimized protocol described before. Amplified DNA fragments were separated in 6% denaturing polyacrilamide gels and visualized by silver –staining according to Creste et al. (2001). Allele scoring was performed using the 10pb DNA ladder (Invitrogen).

The mating system was analyzed according to the mixed mating model of Ritland and Jain (1981) using MLTR software (Ritland 2002), based on the following assumptions: a) each mating event is due to random outcrossing (t) or self-fertilization (with probability $s = 1 - t$); b) the probability of outcrossing is independent of the maternal genotype; c) the pollen pool is homogeneous over all maternal plants; d) there is no selection between fertilization and the time of assay for progeny genotypes; and e) alleles at different loci segregate independently (Ritland and Jain 1981). The following parameters were estimated: multilocus outcrossing rate $(\hat{t}_m)$, single-locus outcrossing rate $(\hat{t}_s)$, outcrossing rate between related individuals $(\hat{t}_m - \hat{t}_s)$, correlation of paternity (r_p) or proportion of full sibs among outcrossed progeny, correlation of the outcrossing rate among loci (r_{ta}) and normalized variation of outcrossing rates among progenies (r_t). All parameters were estimated using maximum likelihood procedures. The number of pollen donors contributing for each progeny was estimated as $1/r_p$ (Ritland 1989). The inbreeding coefficient of maternal parents (F) was also calculated using the MLTR software (Ritland

2002). The standard errors of the reported estimates were calculated based on 10,000 bootstraps, where the progenies were the sampling units.

Genetic Data Analysis (GDA) (Lewis and Zaykin 2000) was used to estimate observed and expected heterozygosities. Allele frequencies and Roger's distance modified by Wright (1978) between progenies were calculated using Tools for Population Genetic Analysis (TFPGA) (Miller 1997). FSTAT (Goudet 2001) was used to estimate the indices of genetic diversity among progenies (Nei's G_{ST}).

Results and Discussion

Development of novel *S. capitata* microsatellite loci

The microsatellite-enriched genomic library of *S. capitata* was suitable to identify microsatellite motifs in the genome of this species (Fig. 1). Of 96 sequenced clones in this library, 67 (70%) contained microsatellites.This frequence is higher than the ones reported for other species (Kim et al. 2007, Ritschel et al. 2004). Since the enrichment used dinucleotide probes, microsatellites with dinucleotide motifs were the most common (75%). Of the 67 sequences containing microsatellites, 52 were suitable for primer design and indicated 79% of primer suitability. Most of the SSRs that were unsuitable for primer design had small flanking regions, which did not allow primer modeling. Some characteristics of the 52 primer pairs are shown in Table 2.

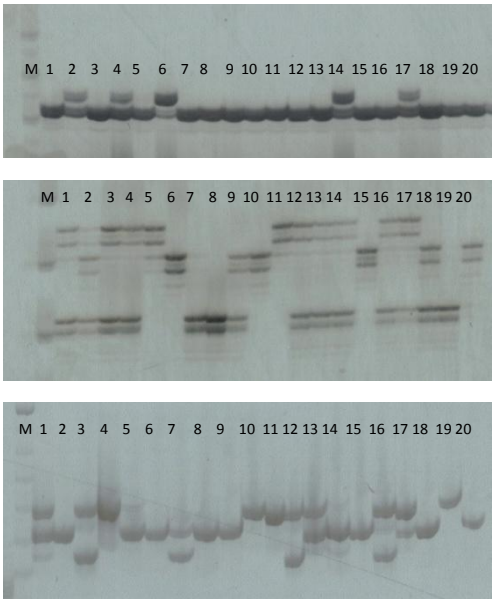

Figure 1 – Amplification pattern of the loci SC 18-01TG9 (A), SC 18-01TH4 (B) and SC 18-01TG12A(C). (M) DNA ladder 10pb, (1-20) *S. capitata* accessions according to Table 1.

PCR primer pairs were screened in 20 accessions of *S. capitata* belonging to Embrapa Beef Cattle, in order to characterize amplification conditions and to evaluate polymorphism. Of 52 developed microsatellites, 14 were polymorphic (27%) and three did not amplify in any of the conditions tested and were discarded (Table 3). No linkage disequilibrium was detected among all loci.

The number of alleles per polymorphic loci in *S. capitata* ranged from two to 9, with an average of 3.3 alelles per locus. In this same set of individuals, Santos et al. (2009a) estimated an average of three alelles per locus using a different set of polymorphic microsatellites. In 20 accessions of *S. guianensis*, Santos et al. (2009b) estimated an average of four alleles per locus. The observed and expected heterozygosities estimated in the present

work varied respectively from 0.10 to 0.55 (average of 0.33) and from 0.18 to 0.77 (average of 0.49). Comparatively, higher values of observed heterozygosity and similar values of expected heterozygosity were observed by Santos et al. (2009a). Moreover, in *S. guianensis* the observed heterozygosity was much lower whereas the expected heterozygosity was similar to the data described here (Santos et al. 2009b). As described for *S. guianensis* (Santos et al. 2009b), the values of observed heterozigosty and expected heterozygosity indicated defficience of heterozygous, which can be associated with the mating system.

Table 2 - Characteristics of the 52 microsatellite loci isolated for *S. capitata*.

Loci Identification	Primer Forward	Primer Reverse	Repeat motif
SC 18-01A2 A	5' AGCAGCATAGGGAATAAAAT 3'	5' CAAAGGCCTAATCAACTGTG 3'	$(TC)_5(AC)_5$
SC 18-01A2 B	5' CTGGACGCATTGTAAGGAAG 3'	5' ATTTTGGGGCTGTGATTTG 3'	$(CA)_3C(TA)_4$
SC 18-01 A5	5' GAACTTGCCACATTTCTCATA 3'	5' AAGGCTTAGGGTTTTCGTT 3'	$(CA)_3(AC)_3...(GA)_{17}$ $(GA)_{17}$
SC 18-01 B4	5' GCTTAGGCCTTATCCAGAA 3'	5' TTGAATTTGTTATTGCTACTACTT 3'	$(GT)_5$
SC 18-01 C1 B	5' AACCCGTTTTCCTTGCTCA 3'	5' CTTTCCACCATAACTTTCTCCT 3'	$(AGA)_4$
SC 18-01 C7 A	5' TTCCGAAATAAGTTGATAATGA 3'	5' GAAAAGATAATGAAAATGTAAATG 3'	$(AAT)_3(AG)_2$
SC 18-01 D1 B	5' GTGTATGAGTGGGTGAGTGAGG 3'	5' GAGTCCCAAGATGAAAAAGAGG 3'	$(GT)_6(TG)_2$
SC 18-01 E4	5' CGGCAACTGGGAAAAATAA 3'	5' ATGGGTAATCACAAATCTTCAG 3'	$(CA)_3CT(CA)_5$
SC 18-01 E10 A	5' AACCCGTTTACCTTGTTCA 3'	5' CTCTTTCCACCATATCTTCCTC 3'	$(AGA)_5$
SC 18-01 E10 B	5' AGAAACGAAGAGAAGAGTGAGA 3'	5' TGTAGAGCAAAAACTAATACCC 3'	$(GT)_6$ $(CT)_6$
SC 18-01 E12	5' TGGTGGTTGTTCTTCATCATC 3'	5' TATGCCATAATTACAAGGAAAC 3'	$(AT)_3(CT)_2$
SC 18-01 G4 A	5' CACGCCCTTGAGTCTGAG 3'	5' ATCTGATGTTTGTGTTCCTATG 3'	$(CA)_9$
SC 18-01 H5	5' GCATCATTTGCATTTGTTTT 3'	5' CTATCACCTCTCCATACCTTATC 3'	$(TG)_9$
SC 18-01 H6 B	5' GGACACGGCAACTTTATCACC 3'	5' CCACCTCACTTGCCACATACC 3'	$(AAAT)_3$ $(GA)_3TA(GA)_2$
SC 18-01 B8	5'-AAGTGGACCAAACCGAGTGA-3'	5' – TGTGTTGGTGGTTTTGTGAAGA – 3'	$(AC)_7$ $(CT)_7$
SC 18-01 E8	5' -CATATCTACGGCCCTAATACTT - 3'	5' - TCCCAACAGCGACAAACTT - 3'	$(AAAT)_4$
SC 18-01T1 - G1	5' - CTGCGTGGTGCCGTATCC - 3'	5' - AGCTCCCCCTTGTCTTCCTCT - 3'	$(AG)_4(CAA)_2$
SC 18-01 T D9-2	5' ACAATAGATGCACCAACAATC 3'	5' GGCTCTCAATTCCTTCCT 3'	$(GA)_3(AG)_2$
SC 18-01T E6	5' CTGTGAGCGTGAGGAGAGT 3'	5' ACATTCACCATTCGACATACAT 3'	$(GTGG)_4$
SC 18-01T F2	5' CTGACCCCACCTAATGAGAAA 3'	5' AGCAAAACAAAACAAACAACACTA 3'	$(TG)_7$
SC 18-01T F6 B	5' CCGTCACCATCTTCCTTCCT 3'	5' TGCCGCAAATCACATCTAAAC 3'	$(GT)_4(TG)_2$ $(GT)_7$
SC 18-01 T F11 B	5' AGGTCTCGCCGCTGCTACTTC 3'	5' ACATGGCGGCGGTTACTCAA 3'	$(GA)_3T(AG)_3$ $(AG)_6$
SC 18-01T G9	5' TCCAGCTAAAGGGCAACACA 3'	5' CCACCGCACACCAGAGATT 3'	$(GAA)_7$
SC 18-01T G12 A	5' ATGCTGATTTTTGGCTCTTTT 3'	5' CCCCTTTTGAACGGATTG 3'	$(ATGGTA)_5$
SC 18-02 F4	5' GGCAAACCCTTATGTATGG 3'	5' GATGTGGGTTGACGAAGTGTA 3'	$(GT)_7$ $(AT)_2(GT)_2(TC)_3$
SC 18-02 F2 A	5' TTCTAATTATCGGGGTATCACA 3'	5' AAACGTCTCCTTCTCCTCTT 3'	$(GGA)_2T(GAA)_3TTC(GAG)_3$
SC 18-02 F1	5' ACTGAATCTTGCGTCTGTTG 3'	5' CGTCGATCCCACTCTCAT 3'	$(AG)_8(GT)_3$ $(GT)_5$ $(AGA)_4AGC(AGA)_3$ $(TTA)_4TGA(TGG)_2(TGA)_2$
SC 18-01T H4	5' GGTATATGGGAGTTCTTGTTCT 3'	5' TTTGTTTGTTTTGCTTTTGTA 3'	$(TG)_{11}$

Table 2 – Continuing.

Loci	Primer F	Primer R	Repeat Motif
SC 18-02 E12	5' AGGGGAAGGGCAAATGGT 3'	5' GCATAGATGGCAAACAGAGACA 3'	$(GT)_5TA(GT)_3$ $(GA)_5CG(CA)_3$ $(CT)_3CA(CT)_{16}$
SC 18-02 E10-1	5' TTTGTATGTGAATGTTTGTGAA 3'	5' AATTATTTACCCGCCTCTTG 3'	$(GT)_{10}$
SC 18-02 E7	5' AAGACAATGCTGCGGAAG 3'	5' TTGATAGAACCACCATTTTG 3'	$(TG)_8$
SC 18-02 E6-1	5' GGCTTTTCGCTCCTCCAG 3'	5' ATCCCGACCCTACTATCATTATG 3'	$(ATT)_4$ $(AGA)_2T(AG)_3$
SC 18-02 E5	5' ACCCTGCAATTTCATCTTCCTC 3'	5' GTCGCACTCTCGCATCCTCAT 3'	$(CA)_7$
SC 18-02 E3-1	5' TTGACAAAGAGCTGAAAAGAA 3'	5' AAATAATGTGCAGTGAATAAAGT 3'	$(GT)_5GA(GT)_3(TG)_3$ $(CA)_3(TA)_2$ $(AAAAC)_3$
SC 18-02 E2	5' GGAAGCCCAAGGAGACAT 3'	5' ACTTCACTATTCAGCGTTTATT 3'	$(AAG)_3G(GA)_2$
SC 18-02 H9	5' TGCCATCCATTCTTTGTT 3'	5' ACGGCGTTTACTTTTGATA 3'	$(GT)_6$
SC 18-02 H11-1	5' GTAGCAGAAACAGCAGAAAATA 3'	5' AAACACCCTTGAAACACTCA 3'	$(AG)_6$ $(AG)_5$ $(GT)_7(CA)_2$
SC 18-02 E1	5' TTTGCCTTGCTTGTTCTG 3'	5' TCCTACTAGTCACTCTGGTTTTA 3'	$(GT)_6$
SC 18-02 H8	5' AAAGTAGAGGGAGATCAAGAATA 3'	5' CTAGCAACAAGGGCATAGA 3'	$(GT)_8C(TG)_3$
SC 18-02 H1	5' GTCATTGTCGTCGTCACC 3'	5' ACCGCATAGCTGTCTTTATT 3'	$(AG)_8G(GA)_6$
SC 18-02 G12-1	5' CATTGTTGTTTGTGGCATTG 3'	5' TTATCCGTTGAAAAAGTGAGTC 3'	$(GT)_8C(TG)_3$
SC 18-02 G5-2	5' CCAAACCGAGTGAAAGGA 3'	5' ATACGAAGGTAACGAAGAAAG 3'	$(CT)_6$ … $(AC)_6$ $(CCA)_3(AC)_4$
SC 18-02 G3-2	5' GTGTAGAACCAACCCAAAGTAG 3'	5' GCAAAGAGCAAACAGAGTATC 3'	$(TTATT)_3$
SC 18-02 F11	5' CGGAGTGAAAGGGCAAGAATG 3'	5' GCTACGGAGAGGAGGACGAGAG 3'	$(AC)_5$
SC 18-02 F10	5' TCATTTCTGCATTTCCTTTTCT 3'	5' AGCGGCCATTTCCCATAA 3'	$(GT)_5(GC)_3$
SC 18-02 F9-2	5' GTGCATTAGTTGATTGATTAGG 3'	5' CATTTTCTTCCCAGTTATTGAT 3'	$(CT)_2(AG)_3C(TTA)_2$
SC 18-02 F9-1	5' ATCCGAGGAATGGTAAGGTAT 3'	5' AAGGACAAACACGAAGACACA 3'	$(AG)_3T(GA)_2$
SC 18-02 F8	5' TTCAATGAAACAGCCTAATG 3'	5' TGACTAAAACCACCCTCACA 3'	$(GT)_6$
SC 18-02 E10-3	5' CTACAATGTATAGTAGGTCCAGTG 3'	5' GTTCATATCCAAAACAGAGCC 3'	$(TA)_2AT(TA)_3$
SC 18-02 E8	5' GTGGTGAAGGTTGGAAGTAG 3'	5' TTTAATAGGCAAAAAGGTGATA 3'	$(AC)_2(CA)_2T(AC)_3$
SC 18-02T G7	5' AGAGAAAGAGGGCAAGTGG 3'	5' AGAAGAATAGAGGGGTGAAAAC 3'	$(CA)_6(CT)_2$
SC 18-025 E11	5' TTAGTGCACAAAGGTCAAGATT 3'	5' GATAAGAAAGCGAAGTCCACA 3'	$(GT)_6(GA)_3(TAA)_2$

Table 3 – Characterization of 49 microsatellites in 20 accessions of *S. capitata*. N - number of alleles, H_O - observed heterozigosity, H_E - expected heterozygosity.

Loci Identification	Product Length (bp)	N	H_O	H_E
SC 18-01 A2A	232-238	2	0.25	0.22
SC 18-01 A5	285-308	6	0.15	0.70
SC 18-01 B4	238-242	3	0.26	0.45
SC 18-01 E10A	242-245	2	0.20	0.18
SC 18-01 E10B	268-272	2	0,10	0.49
SC 18-01 E4	300-310	3	0,35	0.50
SC 18-01 H5	192-196	3	0.41	0.66
SC 18-01T G12A	240-260	3	0.35	0.58
SC 18-01T G9	242-245	2	0.25	0.22
SC 18-01T H4	182-195	3	0.55	0.68
SC 18-01T F2	196-198	2	0.35	0.45
SC 18-02 E12	270-305	9	0.47	0.77
SC 18-02 H1	204-218	4	0.55	0.61
SC 18-02 H9	330-340	2	0.35	0.30
SC18-01 A2B*	241	1	-	-
SC 18-01 C1B*	233	1	-	-
SC 18-01 C7A*	260	1	-	-
SC 18-01 D1B*	180-184	2	-	-
SC 18-01 E12*	245	1	-	-
SC 18-01 G4A*	188-196	2	-	-
SC 18-01 H6B*	295-297	2	-	-
SC 18-01 B8*	170-175	2	-	-
SC18-01 E8*	150	1	-	-
SC 18-01T G1 *	193	1	-	-
SC 18-01T D9*	302	1	-	-
SC 18-01T E6*	182-184	2	-	-
SC 18-01T F6B*	241-243	2	-	-
SC 18-01T F11B*	176	1	-	-
SC 18-02 F4*	196-198	2	-	-
SC 18-02 F2A*	191	1	-	-
SC 18-02 F1*	255	1	-	-
SC 18-02 E10-1*	150-152	2	-	-
SC 18-02 E7*	288	1	-	-
SC 18-02 E6-1*	189	1	-	-
SC 18-02 E5*	230	1	-	-
SC 18-02 E3-1*	225	1	-	-
SC 18-02 E2*	226	1	-	-
SC 18-02 H11-1*	305	1	-	-
SC 18-02 E1*	258	1	-	-
SC 18-02 H8*	197	1	-	-
SC 18-02 G12-1*	210-230	2	-	-
SC18-02 G5-2*	235	1	-	-
SC 18-02 G3-2*	220	1	-	-
SC 18-02 F11*	235	1	-	-

Table 3 – Continuing.

Loci Identification	Product Length (bp)	N	H_O	H_E
SC 18-02 F9-1*	154-158	2	-	-
SC 18-02 F9-2*	179-183	2	-	-
SC 18-02 F8*	175-179	2	-	-
SC 18-025 E11*	222-232	2	-	-

*Monomorphic loci.

Cross-amplification of polymorphic microsatellite loci was tested in *S. guianensis* and *S. macrocephala*. Of 14 polymorphic loci, seven amplified in both species, two amplified only in *S. guianensis*, other two amplified only in *S. macrocephala* and three did not amplify at all (Table 4). *S. macrocephala* is considered a parental genotype of *S. capitata* (Liu et al. 1999). Even though, there was no difference between *S. guianensis* and *S. macrocephala* regarding cross-amplification rates, as also reported by Santos et al. (2009a). In contrast, of 13 *S. macrocephala* microsatellite loci, 11 amplified in *S. capitata* and only six amplified in *S. guianensis* (Santos et al. 2009c). As for 20 *S. guianensis* polymorphic microsatellite loci, only seven amplified in *S. capitata* (Santos et al. 2009b). All cross-amplification reactions were performed using the same conditions described for *S. capitata*. Possibly, the number of transferable microsatellites could be increased by optimization of PCR amplification conditions.

The microsatellites reported here, in conjunction with the ones previous reported, represent a valuable tool for further studies in *S. capitata*. These studies could address the assessment of genetic diversity among germplasm accessions (Huang et al. 2002, Zong et al. 2009) or among commercial

varieties (Landjeva et al. 2006), and studies of natural populations (Tamaki et al. 2009).

Table 4 – Cross-transferability of the 14 polymorphic loci of *S. capitata* to *S. guianensis* and *S. macrocephala.*

Loci	*S. guianensis*	*S. macrocephala*
SC 18-01 A2A	+	+
SC 18-01 A5	+	+
SC 18-01 B4	+	-
SC 18-01 E10A	+	+
SC 18-01 E10B	-	+
SC 18-01 E4	+	+
SC 18-01 H5	+	-
SC 18-01T G12A	-	-
SC 18-01T G9	+	+
SC 18-01T H4	-	-
SC 18-01TF2	-	-
SC 18-02 E12	+	+
SC 18-02 H1	+	+
SC 18-02 H9	-	+

+ successful amplification, - failed amplification

Mating system of *S. capitata* and *S. guianensis*

The outcrossing rates in *S. capitata* and *S.guianensis* were estimated using 20 field-grown plants as maternal parents and 10 individuals per progeny for each species. For *S. capitata*, of 14 polimorphic microsatellite markers, ten were selected based on expected heterozygosity and genotyping quality. For *S. guianensis*, 38 microsatellite loci available in the literature (Vander Stappen et al. 1999b, Santos et al. 2009b) were tested for polymorphism in the 20 maternal plants. Of those 38 microsatellites, five were polymorphic and selected for DNA amplification of 200 individuals of the progeny array.

Among *S. capitata* microsatellite loci (Fig. 2), the number of alleles per locus varied from 2 to 6, with an average of 3 alelles per locus.This result is similar to the observed during the microsatellite development (Table 5). The observed heterozygosity varied from 0.09 to 0.42 (0.25 in average) and the expected heterozygosity ranged from 0.27 to 0.74 (0.49 in average). The expected heterozygosity values were similar to those obtained from the 14 polymorphic markers evaluated in 20 accessions showed above, and indicates that the set of 10 microsatellites selected were able to reveal the same amount of diversity.

Table 5 - Ten loci used to estimate the outcrossing rate in *S. capitata*. N - number of alleles, H_O – observed heterozigosity, H_E - expected heterozygosities in 20 individuals.

Loci Identification	Product Length(bp)	N	H_O	He
SC 18-01 A2A	232-238	2	0.32	0.27
SC 18-01 B4	238-242	2	0.17	0.32
SC 18-01 E4	300-310	3	0.42	0.50
SC 18-01 H5	192-196	2	0.09	0.50
SC 18-01T G12A	240-260	3	0.32	0.61
SC 18-01T G9	242-245	2	0.19	0.27
SC 18-01T H4	182-195	3	0.35	0.65
SC 18-01TF2	196-198	2	0.27	0.39
SC 18-02 H1	204-218	4	0.23	0.62
SC 18-02 E12	270-305	6	0.18	0.74

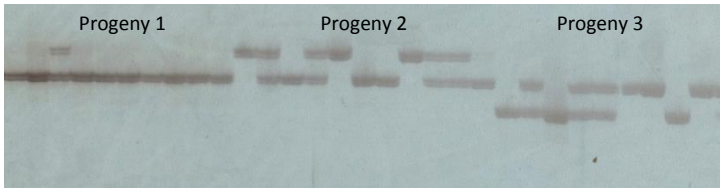

Figure 2 – Pattern of the locus SC18-01G12A in three progenies of the *S. capitata* mating system population.

Among *S. guianensis* microsatellite loci (Fig. 3), the number of alleles varied from 2 to 3, with an average of 2.4 alleles per locus (Table 6). This low number of polymorphic microsatellites and of alleles per locus was expected since breeding genotypes were used and several of them were close related to each other. The observed heterozygosity varied from 0.06 to 0.85 (0.31 in average) and the expected heterozygosity ranged from 0.12 to 0.62 (0.45 in average), showing a deficiency of heterozygous that could indicate some level of autogamy (Barkley et al. 2006).

Table 6 –Polymorphic microsatellites used in the estimation of the mating system in *S. guianensis*. Shown are the loci, the sequence the number of alleles (N), observed (H_O)and expected (H_E).

Loci	N	H_O	H_E
SSR1-9[1]	3	0.18	0.56
SSR4-16a[1]	3	0.25	0.62
SG01D7[2]	2	0.85	0.49
SG03A9[2]	2	0.24	0.44
SG03G8[2]	2	0.06	0.12

[1] Microsatellite developed by Vander Stappen et al. 1999, [2] Microsatellites developed by Santos et al. 2009.

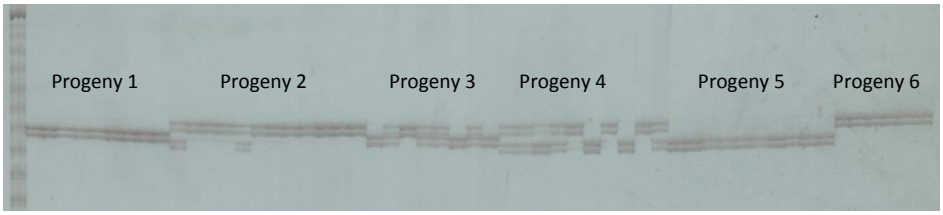

Figure 3 – Pattern of the locus SSR4-16a in six progenies of the *S. guianensis* mating system population.

The *S. capitata* and *S. guianensis* multilocus outcrossing rates were $(\hat{t}_m)0.31\pm0.072$ and $(\hat{t}_m)0.264\pm0.065$ (Table 7) respectively, indicating a mixed mating system with predominance of self-fertilization, while the single-locus estimates were $\hat{t}_s$ was 0.187 ± 0.043 and 0.233 ± 0.061 respectively. The genus *Stylosanthes* is considered to be mainly self-pollinated (Bray and Hutton

1976), but few reports show evidences of outcrossing in some species. Stace (1982) described 2% of outcrossing in *S. scabra* based on an isozime locus. As for *S. guianensis*, Miles (1985) observed about 14% of outcrossing using the color of the flower as genetic marker. In *S. capitata*, Miles (1983) observed 20% of outcrossing studying the presence of hair in the stipules. Outcrossing in *Stylosanthes* depends on the presence and activity of insects. Since the flower color could affect the insect activity, Miles (1985) hypothesized that the data based on this character could show some bias. Vogler and Kalisz (2001) reported that intermediate rates of outcrossing seems to be common in animal-pollinated species, with 49% of those species presenting outcrossing rates between 20 and 80% as observed for these *Stylosanthes* species.

Table 7 – Parameters of the mating system obtained from the analysis of ten microsatellites in 20 progenies of *S. capitata* and *S. guianensis*.

Parameters	**Species**	
	S. capitata	***S. guianensis***
$\hat{t}_m$	0.31(0.072)	0.264(0.065)
$\hat{t}_s$	0.187(0.043)	0.233(0.061)
$\hat{t}_m - \hat{t}_s$	0.123(0.043)	0.031(0.022)
r_p	0.446(0.08)	0.181(0.062)
$1/r_p$	2.24	5.524
r_{ta}	0.86(0.046)	0.916(0.007)
r_t	0.331(0.07)	0.365(0.119)
F_m	0.139(0.084)	0.002(0.026)

The values between brackets refer to standard errors based upon 10,000 bootstraps. $\hat{t}_m$ multilocus outcrossing rate, $\hat{t}_s$ single locus outcrossing rates, r_p multilocus correlation of paternity, r_{ta} correlation of t estimate among loci, r_t correlation of t within progenies and F_m parental coefficient of inbreeding.

The difference between multilocus and singlelocus outcrossing rates represents an estimative of the biparental inbreeding degree, since in its presence $\hat{t}_s$ will be smaller than $\hat{t}_m$ because outcrossing events that are not

detected at a single locus have a higher probability of being detected as more loci are examined (Ritland 1996). This difference $\left(\hat{t}_m - \hat{t}_s\right)$ was significant in both species, being 0.123 ± 0.042 for *S. capitata*, indicating that of the fraction of multilocus outcrossing, of around 12% had occurred between related individuals. For *S. guianensis* this difference was 0.031 ± 0.022 indicating that only 3% of the multilocus outcrossing fraction occurred between related individuals. The correlation of paternity gives the probability that a randomly chosen pair of progenies from the same array were full sibs $\left(r_p\right)$. For *S. capitata*, this probability was 0.446 ± 0.08 and suggested that 14% out of the outcrossing progeny are full sibs $\left(\hat{t}_m r_p\right)$and 17% are half sibs $\left(\hat{t}_m\left(1-r_p\right)\right)$. For *S. guianensis*, there was 0.181 ± 0.062 probability that a randomly chosen pair of progenies from the same array were full sibs $\left(r_p\right)$, which suggested that 5% of the progenies are full sibs $\left(\hat{t}_m r_p\right)$and 21% are half sibs$\left(\hat{t}_m\left(1-r_p\right)\right)$. Even though the progenies were derived from 20 parental plants, only two plants $\left(1/r_p\right)$ contributed as pollen donors in *S. capitata* experiment and about five plants$\left(1/r_p\right)$ contributed as pollen donors in *S. guianensis* experiment. This could be due to limited pollen dispersion and/or nonsynchronous flowering of all individuals (Conte et al. 2008). The correlation of t estimate among all loci (r_{ta}) was high in both species, being 0.86 ± 0.046 among *S. capitata* loci and 0.916 ± 0.007 among *S. guianensis* loci, giving support to the estimates of t. Since both populations were derived from breeding genotypes, several maternal plants were heterozygous leading to inbreeding coefficients (F_m) of the parental generation

smaller than expected for natural populations of those species. The inbreeding coefficient (F_m) of the *S. capitata* parental generation was 0.139 ± 0.08. In *S. guianensis*, F_m was not significantly different from zero (0.002 ± 0.026). For *Oryza glumaepatula*, which presents a higher predominance of autogamy, the estimated F_m varied from 0.399 to 0.846 among diverse populations (Karasawa et al. 2007).

The correlation of t within progenies or the normalized variation of t among progenies (r_t) indicates if the outcrossing rate differs among different progenies. We observed low correlation, being $r_t = 0.331 \pm 0.07$ for *S. capitata* and $r_t = 0.365 \pm 0.119$ for *S. guianensis,* which indicates a small but significant difference in outcrossing rates among progenies. In *S. capitata*, Miles (1983) observed variation of t estimates among progenies (14% to 34%) and among harvesting dates (14% to 47%), which could be due to the insect activity and prolificacy of flowering over time. In *Oryza glumaepatula*, outcrossing rates varied from 0.011 to 0.223 among populations from geographical regions with diverse environmental characteristics (Karasawa et al. 2007). In a natural population of *Araucaria angustifolia*, outcrossing rates varied from 0.822 to 1.121 (Mantovani et al. 2006). The variation observed here is very important for breeding purposes, since even though the species presents a predominance of autogamy, the outcrossing rate can vary among accessions.

Considering *S. capitata,* the genetic distance between progenies averaged 0.52, and ranged from 0.28 (between progenies derived from accessions 12 and 20) to 0.82 (between progenies derived from accessions 13

and 14). Nei's gene diversity (G_{ST}) estimated 52% of genetic diversity between progenies. As for *S.* guianensis, genetic distances between progenies averaged 0.45, ranging from 0.15 (between progenies derived from accessions 9 and 15) up to 0.70 (between progenies derived from accessions 10 and 11) and Nei's gene diversity among progenies (G_{ST}) was 40%. It should be emphasized that the analized individuals for each species were derived from 20 initial plants. This close relatedness could have lead to a G_{ST} value lower than expected for non-related individuals. Even though, the observed G_{ST} values indicated high genetic diversity among progenies, which is in agreement with the predominance of self-fertilization (Hamrick and Godt 1996, Ozkan et al. 2005). Vander Stappen et al. (2000) observed 30% of variation between *S. humilis* from Mexico and from South America, and attributed this high genetic differentiation to ecogeographical isolation and self-pollination.

As previously discussed, the outcrossing estimates of can be variable within a species. In order to overcome this issue and obtain more precise estimates in artificial populations, the assessments must contemplate diverse geographical regions and different years. However, this approach can be time consuming and expensive. The data described in the present work, is the first estimate of outcrossing rate in *S. capitata* and *S. guianensis* inferred from microsatellite markers, which are more accurate than morphological traits (Alcaraz and Hormaza 2007). Hence, this molecular data are highly valuable for breeding and conservation of these species. Since the outcrossing levels were significant in both *S. capitata* and *S. guianensis*, seed multiplication procedures should be reviewed in order to avoid mixture of accessions on

germplasm collections. Moreover, the data demonstrating significant outcrossing rate in these species will allow the enhancement of breeding programs and, consequently, further advance the improvement of *S. capitata* and *S. guianensis*.

References

Alcaraz M, Hormaza J. 2007. Molecular characterization and genetic diversity in an avocado collection of cultivars and local Spanish genotypes using SSRs. Hereditas 144: 244-253.

Azevedo VC, Kanashiro M, Ciampi AY, Grattapaglia D. 2007. Genetic structure and mating system of *Manilkara huberi* (Ducke) A. Chev., a heavily logged Amazonian timber species. The Journal of heredity 98: 646-54.

Barkley NA, Roose ML, Krueger RR, Federici CT. 2006. Assessing genetic diversity and population structure in a citrus germplasm collection utilizing simple sequence repeat markers (SSRs). Theoretical and applied genetics. 112: 1519-31.

Billote N, Risterucci AM, Baurens FC. 1999. Microsatellite enriched libraries: applied methodology for the development of SSR markers in tropical crops. Fruits 54: 277-288.

Bonato AL, Verzignass JR, Resende RM, Fernandes CD, Leguizamon GO. 2002. Extração de DNA genômico de *Stylosanthes* spp. Embrapa Gado de Corte/Comunicado Técnico.

Bray R, Hutton E. 1976. Plant breeding and genetics. Tropical Pasture Research 51: 388-353.

Cameron D, Hutton E, Miles J, Brolmann J. 1984. Plant breeding in *Stylosanthes*. In: Stace, H, Edye, L, The biology and agronomy of *Stylosanthes*. Sydney: Academic Press, 589-606.

Cameron D, Trevorrow R, Liu C. 1997. Recent advances in studies of anthracnose of *Stylosanthes*. II Approachs to breeding for anthracnose resistance in *Stylosanthes* in Australia. Tropical Grasslands 31: 424-429.

Chandra A, Pathak PS, Bhatt RK. 2006. *Stylosanthes* research in India: Prospects and challenges ahead. Current Science 90: 915-921.

Conte R, Sedrez dos Reis M, Mantovani A, Vencovsky R. 2008. Genetic structure and mating system of *Euterpe edulis* Mart. Populations: a comparative analysis using microsatellite and allozyme markers. The Journal of heredity 99: 476-82.

Costa N. 2006. Revisão do género *Stylosanthes*. PhD thesis. Universidade Técnica de Lisboa: 470p.

Creste S, Tulmann Neto A, Figueira A. 2001. Detection of single sequence repeat polymorphisms in denaturing polyacrylamide sequencing gels by silver staining. Plant Molecular Biology Reporter 19: 299-306.

Don RH, Cox PT, Wainwright BJ, Baker K, Mattick JS. 1991. Circumvent spurious priming during gene amplification. Nucleic Acids Research 19: 4008-4008.

Doyle J, Doyle J. 1990. Isolation of plant DNA from fresh tissue. Focus 12: 13-15.

Edye L, Cameron D. 1984. Prospects for *Stylosanthes* improvement and utilization. In: Stace, H, Edye, L, The biology and agronomy of *Stylosanthes*. Sydney: Academic Press, 571-588.

Ferreira M, Costa N. 1979. O genero *Stylosanthes* Sw. no Brasil. Belo Horizonte: EPAMIG.

Gillies AC, Abbott RJ. 1996. Phylogenetic relationships in the genus *Stylosanthes* (Leguminosae) based upon chloroplast DNA variation. Plant Systematics and Evolution 200: 1615-6110.

Goudet J. 2001. FSTAT (Version 1.2): a computer program to calculate F-statistics. Available at: http://www2.unil.ch/popgen/softwares/fstat.htm.

Grof B, Schultze-Kraft R, Miller F. 1979. *Stylosanthes capitata* Vog., some agronomic attributes, and resistance to anthracnose *Colletotrichum gloeosporioides* Penz. Tropical Grasslands 13: 28-37.

Hamrick J, Godt M. 1996. Effects of life history traits on genetic diversity in plant species. Philosophical Transactions of the Royal Society B 351: 1291-1298.

Hendre PS, Phanindranath R, Annapurna V, Lalremruata A, Aggarwal RK. 2008. Development of new genomic microsatellite markers from robusta coffee (*Coffea canephora* Pierre ex A. Froehner) showing broad cross-species transferability and utility in genetic studies. BMC plant biology 8: 51.

Huang Q, Börner a, Röder S, Ganal W. 2002. Assessing genetic diversity of wheat (*Triticum aestivum* L.) germplasm using microsatellite markers. Theoretical and applied genetics. 105: 699-707.

Karasawa M, Vencovsky R, Silva C, Zucchi M, Oliveira G, Veasey E. 2007. Mating system of Brazilian *Oryza glumaepatula* populations studied with microsatellite markers. Annals of botany 99: 245-153.

Kelemu S, Changshun J, Guixi H, Segura G. 2005. Genetic transformation of the tropical forage legume *Stylosanthes guianensis* with rice-chitinase gene confers resistance to *Rhizoctonia* foliar blight disease. African Journal of Biotechnology 4: 1025-1033.

Kim J, Jo BH, Lee KL, Yoon E, Ryu GH, Chung KW. 2007. Identification of new microsatellite loci in *Panax ginseng*. Molecules and Cells 24: 60-68.

Landjeva S, Korzun V, Ganeva G. 2006. Evaluation of Genetic Diversity Among Bulgarian Winter Wheat (*Triticum aestivum* L.) Varieties During the Period 1925–2003 Using Microsatellites. Genetic Resources and Crop Evolution 53: 1605-1614.

Lewis PO, Zaykin D. 2000. Genetic Data Analysis: Computer program for the analysis of allelic data. Available at: http://alleyn.eeb.uconn.edu/gda/ 2000.

Liu C, Musial J, Thomas B. 1999. Genetic relationship among *Stylosanthes* species revealed by RFLP and STS analyses. Theoretical and Applied Genetics 99: 1179-1186.

Mantovani A, Morellato P, Dos Reis M. 2006. Internal genetic structure and outcrossing rate in a natural population of *Araucaria angustifolia* (Bert.) O. Kuntze. Journal of Heredity 97: 466-472.

Maass BL, Sawkins MC. 2004. History, relationships and diversity among *Stylosanthes* species of commercial significance. In: Chakraborty, S, High-yielding anthracnose-resistant *Stylosanthes* for agricultural systems. Sydney: CSIRO, 9-26.

Maass B, Torres A. 1998. Off-types indicates natural outcrossing in five tropical forage legumes in Colombia. Tropical Grasslands 32: 124-130.

Miles J. 1983. Natural outcrossing in *Stylosanthes capitata*. Tropical Grasslands 17: 114-117.

Miles J. 1985. Evaluation of potential genetic marker traits and estimation of outcrossing rate in *Stylosanthes guianensis*. Australian Journal of Agricultural Research 36: 259.

Miller MP. 1997. Tools for population genetic analysis (TFPGA): A Windows program for the analysis of allozyme and molecular population genetic data. Available at: http://www.marksgeneticsoftware.net/tfpga.htm.

O'Conell L, Russel J, Ritland K. 2004. Fine-scale estimation of outcrossing in western redcedar with microsatellite assay of bulked DNA. Heredity 93: 443-449.

Ozkan H, Kafkas S, Ozer M, Brandolini A. 2005. Genetic relationships among South-East Turkey wild barley populations and sampling strategies of *Hordeum spontaneum*. Theoretical and applied genetics. 112: 12-20.

Pathak P, Ramesh C, Bhatt R. 2004. *Stylosanthes* in the reclamation and development of degraded soils in India. In: S, C, High-yielding anthracnose-resistant *Stylosanthes* for agricultural systems. Canberra: Australian Centre for International Agricultural Research, 85-96.

Phaikaew C, Ramesh C, Kexian Y, Stur W. 2004. Utilisation of *Stylosanthes* as a forage crop in Asia. In: Chakraborty, S, High-yielding anthracnose-resistant *Stylosanthes* for agricultural systems. Canberra: CSIRO, 65-76.

Ritland K. 1989. Correlated matings in the partial selfer, *Mimulus guttatus*. Evolution 43: 849-859.

Ritland K. 1996. Estimators for pairwise relatedness and individual inbreeding coefficients. Genetics Research 67: 175–185.

Ritland K. 2002. Extensions of models for the estimation of mating system using n independent loci. Heredity 88: 221-228.

Ritland K, Jain S. 1981. A model for the estimation of outcrossing rate and gene frequencies using n independent loci. Heredity 47: 35-52.

Ritschel PS, Cesar T, Lins L, Louren R, Buso SC, Buso A, Ferreira E. 2004. Development of microsatellite loci from a enriched genomic library for genetic analysis of melon. BMC Plant Biology 14: 1-14.

Santos MO, Sassaki RP, Chiari L, Resende RM, De Souza AP. 2009a. Isolation and characterization of microsatellite loci in tropical forage *Stylosanthes capitata* Vogel. Molecular Ecology Resources 9: 192-194.

Santos MO, Karia CT, Resende RM, Chiari L, Jungmann L, Zucchi MI, Souza AP. 2009b. Isolation and characterization of microsatellite loci in the tropical forage ume *Stylosanthes guianensis* (Aubl.) Sw. Conservation Genetics Resources. Online first, DOI: 10.1007/s12686-009-9010-2.

Santos MO, Sassaki RP, Ferreira THS, Resende RMS, Chiari L, Karia CT, Faleiro FG, Jungmann L, Zucchi MI, Souza AP. 2009c. Polymorphic microsatellite loci for *Stylosanthes macrocephala* Ferr. et Costa, a tropical forage legume. Conservation Genetics Resources. Online first, DOI 10.1007/s12686-009-9112-x.

Stace H. 1982. Breeding systems in *Stylosanthes*. Observations of outcrossing in S. scabra at an alcohol dehydrogenase locus. Australian Journal of Agricultural Research 33: 87-96.

Stace H, Cameron D. 1984. Cytogenetics and the evolution of *Stylosanthes*. In: Stace, H, Edye, L, The biology and agronomy of *Stylosanthes*. Sydney: Academic Press, 49-72.

Tamaki I, Ishida K, Setsuko S, Tomaru N. 2009. Interpopulation variation in mating system and late-stage inbreeding depression in *Magnolia stellata*. Molecular ecology 18: 2365-74.

Temnykh S, DeClerck G, Lukashova A, Lipovich L, Cartinhour S, McCouch S. 2001. Computational and experimental analysis of microsatellites in rice (Oryza sativa L.): frequency, length variation, transposon associations, and genetic marker potential. Genome Research 11: 1441-1452.

Thomas D, Lascano C, Vera R. 1987. A tropical pasture legume for poor soils. SPAN 30: 59-61.

Vander Stappen J, Weltjens I, Volckaert G. 1999b. Microsatellite markers in *Stylosanthes guianensis*. Molecular Ecology 8: 514-517.

Vander Stappen J, Weltjens I, Lopez S, Volckaert G. 2000. Genetic diversity in Mexican *Stylosanthes humilis* as revealed by AFLP, compared to the variabiliy of *S. humilis* accessions of South American origin. Euphytica 113: 145-154.

Vogler D, Kalisz S. 2001. Sex among the flowers: the distribution of plant mating systems. Evolution 55: 202-204.

Williams RJ, Reid R, Schultze-Kraft R, Costa NM, Thomas BD. 1984. Natural distribution of *Stylosanthes*. In: Stace, H, Edye, L, The biology and agronomy of *Stylosanthes*. Sydney: Academic Press, 73-101.

Wright S. 1978. Evolution and the genetics of populations, vol.4. Variability within and among natural populations. Chicago: University of Chicago Press.

Yeh FC, Boyle TJ. 1997. POPGENE. Available at: http://www.ualberta.ca/~fyeh/.

Zong X, Redden RJ, Liu Q, Wang S, Guan J, Liu J, Xu Y, Liu X, Gu J, Yan L, Ades P, Ford R. 2009. Analysis of a diverse global Pisum sp. collection and comparison to a Chinese local *P. sativum* collection with microsatellite markers. Theoretical and applied genetics. 118: 193-204.

6 Artigo III

"Polymorphic microsatellite loci for *Stylosanthes macrocephala* Ferr. et Costa, a tropical forage legume"

Publicado na Conservation Genetics Resources (Online first, DOI 10.1007/s12686-009-9112-x)

Autores: Melissa de O. Santos-Garcia, Rodrigo P. Sassaki, Thaís H. S. Ferreira, Rosângela M. S. Resende, Lucimara Chiari, Cláudio T. Karia, Fábio G. Faleiro, Letícia Jungmann, Maria I. Zucchi, Anete P. Souza.

Conservation Genet Resour
DOI 10.1007/s12686-009-9112-x

TECHNICAL NOTE

Polymorphic microsatellite loci for *Stylosanthes macrocephala* Ferr. et Costa, a tropical forage legume

M. O. Santos · R. P. Sassaki · T. H. S. Ferreira · R. M. S. Resende · L. Chiari · C. T. Karia · F. G. Faleiro · L. Jungmann · M. I. Zucchi · A. P. Souza

Received: 15 September 2009 / Accepted: 24 September 2009

Abstract Microsatellites were developed for *Stylosanthes macrocephala*, aiming at developing tools for studying the genetic diversity of this species. A total of 13 polymorphic microsatellite markers were isolated from a *S. macrocephala* enriched genomic library. The isolated microsatellites were characterized in 20 accessions of the *S. macrocephala* germplasm collection belonging to the Empresa Brasileira de Pesquisa Agropecuária (Embrapa) Cerrados. The number of alleles per locus varied from 2 to 11, with an average of 4 alleles per locus. The observed and expected heterozygosities ranged from 0 to 0.25 and 0.05 to 0.90, respectively. Cross-amplification of the *S. macrocephala* polymorphic microsatellites was evaluated in three other *Stylosanthes* species. The microsatellites reported herein are the first set of microsatellite markers developed for *S. macrocephala* and are potentially useful for further studies on genetic diversity, conservation and breeding of this species.

M. O. Santos · R. P. Sassaki · T. H. S. Ferreira · A. P. Souza (✉)
Centro de Biologia Molecular e Engenharia Genética, Universidade Estadual de Campinas, CP 6010, Campinas 13083-970, SP, Brazil
e-mail: anete@unicamp.br

R. M. S. Resende · L. Chiari · L. Jungmann
Empresa Brasileira de Pesquisa Agropecuária (Embrapa) – Gado de Corte, BR 262, Km 4, Campo Grande 79002-970, MS, Brazil

C. T. Karia · F. G. Faleiro
Empresa Brasileira de Pesquisa Agropecuária (Embrapa) – Cerrados, BR 020, Km 18, Planaltina 73310-970, DF, Brazil

M. I. Zucchi
Pólo Apta Centro Sul – Rod, SP 127 km 30, CP 28, Piracicaba CEP 13400-970, SP, Brazil

Published online: 04 October 2009

Keywords Molecular markers · Genetic diversity · Genomic enriched library

The genus *Stylosanthes* Sw. (Fabaceae) is widely distributed in the tropical areas of America, Africa and Southeast Asia (Williams et al. 1984; Costa 2006). Of the 48 *Stylosanthes* species, 45% are found in Brazil, which is the major center of origin and diversity of this genus (Costa 2006). *Stylosanthes* species grow relatively well in poor soil and have high protein contents. Hence, they have been used for soil conservation and improvement through nitrogen fixation, and for recovery of degraded wastelands (Baldión et al. 1975; Kelemu et al. 2005). Several *Stylosanthes* species are important pasture legumes in tropical and subtropical regions (Edye and Cameron 1984).

Stylosanthes macrocephala is a diploid ($2n = 20$) species and belongs to the Styposanthes Section (Mannetje 1984). In Brazil, it occurs in sandy soils of the Cerrado and Caatinga regions (Costa 2006) and some ecotypes are tolerant to anthracnose (*Colletotrichum gloeosporioides*) (Costa and Ferreira 1984). *Stylosanthes macrocephala* has been considered one of the putative parental species of the allotetraploid *S. capitata*, which is one of the most important tropical forage legumes (Liu et al. 1999; Vander Stappen et al. 1999). To our knowledge, specific microsatellite markers have not yet been developed for *S. macrocephala*. Here, we report the isolation and characterization of polymorphic microsatellites for *S. macrocephala* that are potentially useful for genetic studies of this species.

Samples of total genomic DNA were isolated from *S. macrocephala* fresh leaves using the cetyltrimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1990) with modifications (Bonato et al. 2002). A genomic

Table 1 Characteristics of 26 microsatellite loci of *Stylosanthes macrocephala*

Locus name	GenBank accession no.	Forward and reverse primers (5′–3′)	Repeat motif	No. of alleles	Product size range (bp)	H_O	H_E	*P* value* HWE
SM02A5	GQ463439	5′ACACATCAACCGAGTCACAAA3′ 5′AGGGGGAATAAAGCCAACAT3′	$(CA)_6$…$(CA)_6$	2	296–300	0	0.10	0.026
SM02A10	GQ463440	5′ATAAAGAAAACGCGAACAAAT3′ 5′ACAACCCAACATCATCATCC3′	$(AG)_{10}$…$(CTT)_5$…$(GT)_5$	3	298–340	0.05	0.14	0.077
SM01D3	GQ463441	5′CCACATATAGCTTTCCCATTCC3′ 5′CCTTTTCCGTGATTTTGTCTCT3′	$(CA)_7$	2	205–207	0	0.10	0.026
SM02A2	GQ463442	5′TACTACTTGCTTTTACCCTCTC3′ 5′GCCCTCTGCCTTCCATT3′	$(CT)_{15}$	11	270–300	0.20	0.90	0.002*
SM01B11	GQ463443	5′TGCGACAGTTGGGTAGTAG3′ 5′AGCCTTTCTCTTATTTATCTTT3′	$(TC)_8$…$(CT)_9$	7	286–315	0	0.72	0.000*
SM02C9	GQ463444	5′GCTTTTCGGGTATGCTTGTA3′ 5′GAAACGGAACTATTGAGATTGA3′	$(GT)_7$	3	108–112	0.10	0.10	1.000
SM02G2	GQ463445	5′CAGAAATGGGGAATGAAGA3′ 5′CGTTTGCCCGTATCGTAAG3′	$(TC)_5(AC)_5$	2	261–263	0.10	0.41	0.004
SM02G5	GQ463446	5′CTGACAAAGCCGTAGGAGACT3′ 5′AATGGGTGGAAGCAAAAGAC3′	$(GT)_6$…$(GA)_8$…$(GA)_5$	5	235–260	0.05	0.67	0.000*
SM01B5	GQ463447	5′AGCTTACTGTTGGGGTGTCTA3′ 5′TTTTCCGTGATTTTGTCTCTGT3′	$(CA)_7$	2	248–250	0.05	0.05	1.000
SM01B6	GQ463448	5′AATAACAAAAAGATCGCCACAT3′ 5′CCCCCTTAATTCCTCCACTC3′	$(TG)_7$	3	273–277	0.05	0.23	0.010
SM02A8	GQ463449	5′TTGTTATTTTTTTATGGGAGCA3′ 5′GTTATGTGATGGATTGAGATGT3′	$(GT)_7$	3	261–265	0.25	0.58	0.002*
SM02A9	GQ463450	5′CAATATGGGAAGTGACAAATGC3′ 5′GGTAAATAAGAAAGAAAGCCTT3′	$(TG)_7$…$(AT)_5$…$(TA)_6$	3	251–257	0.16	0.24	0.259
SM02G3B	GQ463451	5′AAAAGAGAAGGCGAGAAGGGAA3′ 5′GGAGGAGGTGGAGATTGGAGAC3′	$(CA)_4(CT)_8$…$(CA)_5$	8	292–340	0.21	0.84	0.000*
SM02B9[a]	GQ919188	5′GGGTGAAACTCGAACCCTTACC3′ 5′GGCAAAACCAGCACCCTCAAC3′	$(GT)_7$	1	294	–	–	–
SM02B1[a]	GQ919189	5′CGAGCGCGAACACCTACG3′ 5′AGCTCCGGATCAAAAGTTATGG3′	$(CA)_7$	1	230	–	–	–
SM02D6[a]	GQ919190	5′TAAACTCACGGCACCCTCA3′ 5′ATCAACAATCAACAACCAAATG3′	$(AC)_{10}$	1	292	–	–	–
SM02D5[a]	GQ919191	5′TGGCTATGCACAACAACC3′ 5′ATAGCTTCCACATCTCTTACAG3′	$(AAC)_4$	1	130	–	–	–
SM02B6[a]	GQ919192	5′CGGCGTTGTGGCGTAGC3′ 5′GGTGGGGGGCGGTGAGAG3′	$(AC)_5$	1	176	–	–	–

Table 1 continued

Locus name	GenBank accession no.	Forward and reverse primers (5′–3′)	Repeat motif	No. of alleles	Product size range (bp)	H_O	H_E	*P* value* HWE
SM02E3[a]	GQ919193	5′ATATGGACAAGGATTAGGAGAA3′ 5′ATTAAGCGTGGGTGAGGTG3′	$(CA)_7$	1	291	–	–	–
SM02E10A[a]	GQ919194	5′CGGGCTTTTGGGATTGAGTTA3′ 5′TCAGGGCATTTGGTGATTATGA3′	$(TGA)_4$	1	205	–	–	–
SM02D7[a]	GQ919195	5′TGCGTCTTCATCATGTTCTTCT3′ 5′GAGCCGATAAATACTTCTTCCT3′	$(TTTA)_4$	1	218	–	–	–
SM01C2[a]	GQ919196	5′GCAGTGTTGTGGCGTAGC3′ 5′AAACCGATTAAAATGAAGAGAA3′	$(AC)_7...(CT)_5$	1	273	–	–	–
SM02F12[a]	GQ919197	5′ATTAGTGCTTTGATGGGATTTA3′ 5′ATGGTATATTGGCGTGGAT3′	$(TG)_6$	1	269	–	–	–
SM02C12[a]	GQ919198	5′GCGATCGGAGTTTGTTTGA3′ 5′AGCCCCTTAAGTGAGTGTGC3′	$(GT)_5$	1	176	–	–	–
SM01A12[a]	GQ919199	5′CCCCCACCTTCACCACCATCA3′ 5′GGACACGGACGCGAACACCTAT3′	$(GT)_6$	1	184	–	–	–
SM02C10[a]	GQ919200	5′GCGATATAACCCCAACCA3′ 5′CTCCTTTCTTTTCCGTGTG3′	$(AT)_6...(AC)_6$	1	263	–	–	–

H_O Observed heterozygosity; H_E Expected heterozigosity

* Departing significantly from HWE at $P < 0.05$ after Bonferroni correction

[a] Monomorphic loci

enriched library was constructed according to Billotte et al. (1999). Samples of purified total DNA were digested with *Rsa* I, hybridized with biotin-labelled $(CT)_8$ and $(GT)_8$ repeats and captured using the kit Streptavidin Magne-Sphere Paramagnetic Particles (Promega). Captured fragments were amplified by polymerase chain reaction (PCR), ligated into pGem-T Easy (Promega) and then transformed into competent XL1-blue *Escherichia coli* cells. Cultures were incubated on HM/FM medium supplemented with ampicillin, IPTG and X-galactosidase. A total of 72 recombinant clones were selected and bi-directionally sequenced in an automated sequencer ABI PRISM® 377 using T7 and SP6 primers and the v3.1 Big Dye terminator kit (PerkinElmer Applied Biosystems. DNA sequences were assembled and edited with Seqman (DNAStar). Repetitive regions were identified using the Simple Sequence Repeat Identification Tool (Temnykh et al. 2001). Of the 72 *S. macrocephala* sequenced clones, 26 contained microsatellite sequences with more than five repeats and flanking regions suitable for primer design. The Primer Select (DNAStar) software was used to design 26 primer pairs complementary to the flanking regions of those repetitive DNA fragments.

The isolated microsatellite loci were evaluated for polymorphism in 20 *S. macrocephala* accessions of the germplasm collection belonging to Empresa Brasileira de Pesquisa Agropecuária (Embrapa) Cerrados. DNA samples were isolated from bulks of three plants using the CTAB method (Doyle and Doyle 1990) with few modifications (Faleiro et al. 2003). PCR amplifications were performed in a total reaction volume of 15 µl, consisting of 1× PCR buffer, 1.5 mM $MgCl_2$, 0.25 mM of each dNTP (Invitrogen), 0.8 µM of each primer, 1U *Taq* DNA polymerase (Invitrogen) and 10 ng of genomic DNA. Reactions were carried out on a PTC-200 (MJ Research) thermocycler programmed for an initial denaturation at 94°C for 1 min, followed by 30 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and a final extension at 72° for 5 min. The amplification products were resolved by electrophoresis on 6% denaturing polyacrylamide gels, at 75 W for about 120 min. DNA fragments were silver stained (Creste et al. 2001) and the alleles were scored using the 10 pb DNA Ladder (Invitrogen).

Of 26 loci, 13 were polymorphic showing intense and well defined DNA bands. The number of alleles per locus ranged from 2 to 11, with an average of 4 alleles per locus. The Tools for Genetic Population Analysis (TFPGA) was used for descriptive statistics and test of Hardy–Weinberg Equilibrium (Miller 1997). The observed heterozygosity ranged from 0 to 0.25 and averaged 0.09, while the expected heterozygosity varied from 0.05 to 0.90 with an average of 0.40. The observed heterozygosity is low in *S. macrocephala*, as it is for *S. guianensis* (Santos et al. 2009), suggesting predominance of autogamy in both species. Of the 13 polymorphic microsatellite loci, five deviated significantly from the Hardy–Weinberg Equilibrium ($P < 0.05$) after Bonferroni correction (Table 1). The linkage disequilibrium analysis using Genepop 1.2 (Raymond and Rousset, 1995) did not detected disequilibrium among all loci. Cross-species amplifications were evaluated in the species *Stylosanthes capitata*, *S. guianensis* and *S. pilosa*. Eleven of the 13 polymorphic loci cross-amplified in *S. capitata*, 12 amplified in *S. pilosa* and only six loci amplified in *S. guianensis* (Table 2).

Table 2 Cross-species amplification of 13 polymorphic microsatellite loci of *Stylosanthes macrocephala* in *S. capitata*, *S. guianensis* and *S. pilosa*. Successful amplification (+) and no amplification (−) are indicated

Locus	*S. capitata*	*S. guianensis*	*S. pilosa*
SM02A5	+	+	+
SM02A10	+	−	+
SM01D3	+	+	+
SM02A2	+	+	+
SM01B11	+	+	+
SM02C9	+	−	+
SM02G2	+	−	+
SM02G5	+	+	+
SM01B5	+	−	+
SM01B6	+	+	−
SM02A8	−	−	+
SM02A9	−	−	+
SM02G3B	+	−	+

The data demonstrated that the microsatellites developed for *S. macrocephala* are potentially useful for studies on genetic diversity of germplasm collections and for natural population genetic studies.

Acknowledgments The authors are grateful to Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for financial support to this research (Grant # 2005/51010-0). M.O.S. received a scholarship from FAPESP (2005/52211-9) and A.P.S. is recipient of a research fellowship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

References

Baldión R, Lozano JC, Grof B (1975) Evaluación de la resistência de *Stylosanthes* spp. a la antracnosis (*Colletotrichum gloeosporioides*). Fitopatologia 10:104–108

Billotte N, Lagoda PJR, Risterucci AM, Baurens FC (1999) Microsatellite-enriched libraries: applied methodology for the development of SSR markers in tropical crops. Fruits 54:277–288

Bonato ALV, Verzignassi JR, Resende RMS, Fernandes CD, Leguizamón GOC (2002) Extração de DNA genômico de

Stylosanthes spp. Comunicado Técnico 78. Empresa Brasileira de Pesquisa Agropecuária, EMBRAPA

Costa NMS (2006) Revisão do Género *Stylosanthes* Sw. PhD Thesis. Lisboa, 470 p

Costa NMS, Ferreira MB (1984) Some Brazilian species of *Stylosanthes*. In: Stacce HM, Edye LA (eds) The biology and agronomy of *Stylosanthes*. Academic Press, Sydney, pp 23–48

Creste S, Tulmann Neto A, Figueira A (2001) Detection of single sequence repeat polymorphisms in denaturing polyacrylamide sequencing gels by silver staining. Plant Mol Biol Rep 19:299–306

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12:13–15

Edye LA, Cameron DG (1984) Prospects for *Stylosanthes* improvement and utilization. In: Stace HM, Edye LA (eds) The biology and agronomy of *Stylosanthes*. Academic Press, Sydney, pp 571–587

Faleiro FG, Faleiro ASG, Cordeiro MCR, Karia CT (2003) Metodologia para extração de DNA de espécies nativas do cerrado visando análises moleculares. Comunicado Técnico 92. Empresa Brasileira de Pesquisa Agropecuária, EMBRAPA

Kelemu S, Changshun J, Guixi H, Segura G (2005) Genetic transformation of the tropical forage legume *Stylosanthes guianensis* with a rice-chitinase gene confers resistance to *Rhizoctonia* foliar blight disease. Afr J Biotechnol 10:1025–1033

Liu CJ, Musial JM, Thomas BD (1999) Genetic relationships among *Stylosanthes* species revealed by RFLP and STS analyses. Theor Appl Genet 99:1179–1186

Mannetje Lt' (1984) Considerations on the taxonomy of the genus *Stylosanthes*. In: Stace HM, Edye LA (eds) The biology and agronomy of *Stylosanthes*. Academic Press, Sydney, pp 1–21

Miller MP (1997) Tools for population genetic analysis (TFPGA) 1.3. A windows program for the analysis of allozyme and molecular population genetic data. Computer software distributed by author

Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. J Hered 86:248–249

Santos MO, Karia CT, Resende RMS, Chiari L, Jungmann L, Zucchi MI, Souza AP (2009) Isolation and characterization of microsatellite loci in the tropical forage legume *Stylosanthes guianensis* (Aubl.) Sw. Conserv Genet Resour. doi:10.1007/s12686-009-9010-2

Temnykh S, DeClerck G, Lukashova A, Lipovich L, Catinhour S, McCouch S (2001) Computational and experimental analysis of microsatellites in rice (*Oryza sativa* L.): frequency, length variation, transposon associations, and genetic marker potential. Genome 11:1441–1452

Vander Stappen J, Weltjens I, Van Campenhout S, Volckhart G (1999) Genetic relationships among *Stylosanthes* species as revealed by sequence-tagged site markers. Theor Appl Genet 98:1054–1062

Williams RJ, Reid R, Shultze-Kraft R, Costa NMS, Thomas BD (1984) Natural distribution of *Stylosanthes*. In: Stacce HM, Edye LA (eds) The biology and agronomy of *Stylosanthes*. Academic Press, Sydney, pp 73–101

7 Artigo IV

"Tropical Forage Legumes: II Genetic Diversity in *Stylosanthes macrocephala* Ferr. et Costa"

A ser submetido à revista Theoretical and Applied Genetics

Autores: Melissa de O. Santos-Garcia, Rodrigo P Sassaki, Thais H S Ferreira, Rosângela M. S. Resende, Lucimara Chiari, Claudio T. Karia, Fabio G Faleiro, Maria I. Zucchi, Anete P. Souza.

Tropical Forage Legumes: II Genetic diversity in *Stylosanthes macrocephala* Ferr. et Costa

M O Santos-Garcia[1]; R P Sassaki[1]; T H S Ferreira[1]; R M S Resende[2]; L Chiari[2]; C T Karia[3]; F G Faleiro[3]; M I Zuchi[4]; A P Souza[1,5]

[1] Centro de Biologia Molecular e Engenharia Genética, Universidade Estadual de Campinas, CP 6010, Campinas, SP - Brazil. 13083-970

[2] Empresa Brasileira de Pesquisa Agropecuária (Embrapa) - Gado de Corte. BR 262, Km 4. Campo Grande, MS, Brazil. 79002-970.

[3] Empresa Brasileira de Pesquisa Agropecuária (Embrapa) – Cerrados. BR 020, Km 18. Planaltina, DF – Brazil. 73310-970

[4] Pólo Apta Centro Sul – Rod. SP 127 km 30, Piracicaba – SP, Brazil, CP 28, CEP 13400-970.

[5] Departamento de Biologia Vegetal, Instituto de Biologia, Universidade Estadual de Campinas, CP 6109, Campinas 13083-970, SP, Brazil.

Keywords: Molecular markers, plant breeding, population structure, germplasm collection

*Corresponding author: Anete Pereira de Souza. Centro de Biologia Molecular e Engenharia Genética. Universidade Estadual de Campinas, Campinas, SP, Brazil. CP 6010. CEP 13083-970.

E-mail: anete@unicamp.br
Phone: 55-19-3521-1132
FAX: 55-19-3521-1089

Abstract

Stylosanthes macrocephala is an important forage legume for tropical and subtropical areas. The Brazilian Agricultural Research Corporation Cerrados (Embrapa-Cerrados) has a *S. macrocephala* germplasm collection that comprises several accessions potentially useful for breeding programs. In the present work, 13 polymorphic microsatellite markers were used for assessing the genetic diversity of the Embrapa-Cerrados germplasm collection, aiming at generating information that could be useful for the improvement of *S. macrocephala*. The average number of alleles per locus was 4.7, varying from two to 11. The means of observed and expected heterozygosities were respectively 0.08 and 0.36, showing a deficit of heterozygous. The Roger's genetic distance was calculated and varied from 0 to 0.83 and revealed genetic diversity higher than that previously reported for this germplasm collection using RAPD markers. The model-based clustering method implemented in the software STRUCTURE distinguished five groups among *S. macrocephala* accessions. The Nei's gene diversity showed that 27% of the diversity was due to genetic differences among the accessions groups. The data presented herein demonstrate a high degree of genetic diversity in the Embrapa-Cerrados germplasm collection of *S. macrocephala*, which can be exploited in breeding programs.

Introduction

The genus *Stylosanthes* Sw. is a member of the family Fabaceae, tribe Aeschynomeneae, subtribe Stylosanthinae and includes 48 herbaceous-subshrub species of Neotropical origin (Costa 2006). Most *Stylosanthes* species are found in tropical, subtropical and warm temperate areas of Americas, while few are distributed in the southeastern part of the United States, the Antilles, the Galapagos Islands, central and southern Africa, Madagascar, southern India and Ceylon (Williams et al. 1984). The major diversity center is Central Brazil (Ferreira and Costa 1979, Stace and Cameron 1984), which comprises 45% of all species of the genus, and where the greatest phenotypic variation and endemism are observed (Costa 2006). A second diversity center is located in Central America, in the region formed by Mexico and Caribbean Islands (Stace and Cameron, 1984).

Several *Stylosanthes* species are important as source of pasture legume for tropical and subtropical environments (Edye and Cameron 1984), being also used for soil improvement through nitrogen fixation and for the reclaiming of degraded wastelands. In China and Thailand, other uses have been described such as the utilization as cut-and-carry fodders (Kelemu et al. 2005, Pathak et al. 2004 , Phaikaew et al. 2004). In Brazil, seven cultivars were recommended or released on the basis of evaluation and selection in Brazilian conditions, and they all belong to one of the species *S. capitata*, *S. guianensis* or *S. macrocephala*, although just two of them are presently available in the market (Andrade et al. 2004, Karia 2008).

Stylosanthes macrocephala is a diploid species (2n=20) that belongs to the Styposanthes Section (t' Manetje 1984). This species occurs in sandy soils of the Brazilian regions of Cerrado and Caatinga (Costa 2006) and several of the ecotypes show tolerance to anthracnose (*Colletotrichum gloeosporioides*), which is the most important disease that affects this genus (Costa and Ferreira 1984). *S. macrocephala* is considered to be the maternal parental of the allotetraploid *S. capitata*, another important tropical forage legume (Gillies and Abbott 1996, Liu et al. 1999).

The Brazilian Agricultural Research Corporation – Embrapa Cerrados Research Center (Embrapa-Cerrados) has a germplasm collection of 134 accessions of *S. macrocephala*, in which several are potentially useful for breeding programs (Barros et al. 2005, Andrade et al. 2004). Further evaluation of the amount and distribution of genetic diversity among the accessions can highly enhance the genetic exploitation of this germplasm collection (Struss and Plieske 1998, Ferriol et al. 2004, Blair et al. 2007). Molecular markers, along with morphoagronomical data and ecological characteristics of the sites where the accessions were sampled has proved suitable for evaluating germplasm, organizing genetic resources, identifying putative duplicated accessions, determining how variation is distributed among accessions and constructing smaller germplasm collections (Westman and Kresovich 1997, Zong et al. 2009). Microsatellites or simple sequence repeats (SSRs) have shown to be one of the most suitable markers for such purposes (Huang et al. 2002, Hao et al. 2006, Landjeva et al. 2006). Microsatellites are abundant, evenly distributed in the chromosomes, codominant and multiallelic.

Since knowledge about the genetic diversity and structure of germplasm collections can be an important foundation for breeding programs, we used polymorphic microsatellite markers to evaluate the Embrapa-Cerrados germplasm collection of *S. macrocephala*.

Material and Methods

DNA Extraction and Polymerase Chain Reaction (PCR)

A total of 134 accessions of *S. macrocephala* from Embrapa-Cerrados (Table 1) were evaluated using 13 microsatellites developed by Santos et al. (2009c).

DNA samples were extracted from fresh leaves using the cetyltrimethyl ammonium bromide (CTAB) extraction method as described by Faleiro et al. (2003). Leaves from three different plants per accession were bulked for DNA extraction. PCR amplifications were carried out on a PTC-200 (MJ Research) thermo-cycler with a 20 µl final reaction volume consisting of 1X PCR buffer, 1.5 mM $MgCl_2$, 0.25 mM of each dNTP (Invitrogen), 0.8 µM of each primer, 1U de *Taq* DNA polymerase (Invitrogen) and 20ng of genomic DNA. The amplification protocol consisted of an initial denaturation at 94ºC for 1 min followed by 30 cycles of 94ºC for 1 min, annealing at 60ºC for 1 min and 72ºC for 1 min and a final extension at 72º for 5 min. The PCR amplified DNA fragments were separated by eletrophoresis on 6% denaturing polyacrilamide gels running at 75W for about 120 min. The polyacrylamide gels were then

silver stained (Creste et al. 2001) and the allele scoring was done in comparison with a 10pb DNA ladder (Invitrogen).

Table 1 – List of 134 accessions of *S. macrocephala* from the Embrapa-Cerrados germplasm collection that were analysed by microsatellite markers. The sampling region refers to States of Brazil where the germplasm accessions were sampled. The group refers to the Bayesian analysis.

Code	CPAC	Region	Group	Code	CPAC	Region	Group
1	139	Distrito Federal	D	29	1307	Distrito Federal	D
2	1030	n.a	E	30	1308	Goiás	B
3	1031	Goiás	E	31	1309	Distrito Federal	D
4	1032	Goias	A	32	1310	Distrito Federal	D
5	1033	Bahia	A	33	1311	Goiás	D
6	1036	Bahia	A	34	1332	Bahia	E
7	1037	Bahia	C	35	1333	Bahia	A
8	1039	Bahia	A	36	1335	n.a	C
9	1040	Bahia	D	37	1337	Bahia	B
10	1043	Bahia	A	38	1339	Bahia	C
11	1190	Minas Gerais	E	39	1340	Bahia	C
12	1191	Minas Gerais	E	40	1341	Bahia	B
13	1192	Minas Gerais	E	41	1345	Bahia	C
14	1193	Minas Gerais	E	42	1346	Bahia	A
15	1194	Minas Gerais	B	43	1347	Bahia	C
16	1196	Minas Gerais	D	44	1367	Goiás	C
17	1197	Minas Gerais	B	45	1370	n.a	C
18	1198	Minas Gerais	E	46	1373	Bahia	C
19	1200	Minas Gerais	E	47	1376	Bahia	B
20	1201	Minas Gerais	B	48	1378	Bahia	E
21	1202	Minas Gerais	E	49	1382	Bahia	A
22	1204	Minas Gerais	B	50	1383	Bahia	A
23	1205	Minas Gerais	B	51	1636	Bahia	D
24	1206	Minas Gerais	E	52	1639	Bahia	C
25	1303	n.a	B	53	1640	Bahia	D
26	1304	n.a	B	54	2227	Bahia	D
27	1305	n.a	B	55	2229	n.a	D
28	1306	n.a	B	56	2230	Goiás	A

Table 1 – Continuing.

Code	CPAC	Region	Group	Code	CPAC	Region	Group
57	2231	Bahia	A	96	4378	n.a	A
58	2239	Bahia	B	97	5184	n.a	B
59	2254	n.a	D	98	5296	n.a	E
60	2255	n.a	B	99	1362ª	n.a	C
61	2256	n.a	C	100	208/479	n.a	D
62	2257	n.a	B	101	1336	Bahia	A
63	2258	n.a	E	102	1363	Bahia	B
64	2259	n.a	E	103	1361	Bahia	C
65	2260	n.a	E	104	1358	Bahia	C
66	2261	n.a	C	105	2251	Minas Gerais	B
67	2262	n.a	E	106	2252	Bahia	C
68	2263	n.a	E	107	2709	n.a	D
69	2265	Minas Gerais	E	108	2264	n.a	B
70	2266	n.a	E	109	2717	Bahia	B
71	2267	n.a	B	110	2790	Bahia	E
72	2710	n.a	D	111	2792	Bahia	C
73	2711	n.a	A	112	4135	n.a	C
74	2712	Piaui	C	113	4137	n.a	D
75	2713	Bahia	D	114	1041	Bahia	C
76	2714	Bahia	C	115	1189	n.a	D
77	2715	Bahia	C	116	1045	Bahia	C
78	2716	Bahia	C	117	1199	Minas Gerais	E
79	2719	Bahia	C	118	1187	Bahia	C
80	2720	Bahia	E	119	1302	n.a	D
81	2777	Distrito Federal	D	120	1380	n.a	C
82	2778	n.a	E	121	1641	Goiás	D
83	2782	Bahia	D	122	1642	Goiás	D
84	2783	Bahia	C	123	1646	n.a	D
85	1035	Bahia	C	124	2232	Bahia	A
86	2795	Minas Gerais	B	125	2235	Bahia	A
87	4136	n.a	D	126	2268	n.a	B
88	4138	n.a	C	127	2721	Bahia	C
89	4139	n.a	C	128	2779	Goiás	A
90	4140	n.a	D	129	2780	Goiás	A
91	4166	n.a	D	130	2784	Bahia	A
92	4167	n.a	D	131	2789	n.a	D
93	4168	n.a	D	132	2794	Minas Gerais	B
94	4200	n.a	D	133	4377	n.a	A
95	4271	n.a	D	134	4971	n.a	D

Data Analysis

The Genetic Data Analysis (GDA) (Lewis and Zaykin 2000) was used to estimate the observed and expected heterozygosities. Allele frequencies and Roger's distance modified by Wright (1978) were calculated between all pairs of accessions using Tools for Population Genetic Analysis (TFPGA) (Miller 1997).

The software STRUCTURE 2.0 (Pritchard et al. 2000) was utilized to infer population structure and assign accessions to populations based on the SSR genotypes. Structure uses model-based clustering in which a Bayesian approach identifies clusters based on fit to Hardy-Weinberg equilibrium and linkage equilibrium. The optimum number of populations (K) was selected after ten independent runs of a burn-in period of 300,000 and 400,000 replications using a model that allows for admixture and correlated allele frequencies and testing K=2 to K=20. Identification of number of distinct clusters (K) was performed according to Evanno et al. (2005). The DARwin 5.0 software (Perrier and Jacquemoud-Collet 2006) was used to define the genetic relationships among accessions based on the Roger's genetic distance and Neighbor-joining clustering method. FSTAT (Goudet 2001) was used to calculate Nei's G_{ST} among the groups formed by the STRUCTURE analysis.

Results and Discussion

The 13 microsatellite loci used in the present work were suitable for evaluating the 134 accessions of *S. macrocephala* (Fig. 1). The number of alleles per locus ranged from two to 11 and averaged 4.7 alleles per locus (Table 2). The same range, with the smaller average of 4 alelles per locus was observed by Santos et al. (2009c) studying 20 accessions of this same germplasm collection. As for *S. capitata* the observed range of alleles per locus varied from two to seven alleles per locus and averaged 3.3 in 20 accessions analysed with eight microsatellites (Santos et al. 2009a). In *S. guianensis*, the analysis of 20 accessions using 20 loci revealed number of alleles between two and seven, with an average of four (Santos et al. 2009b). Even though, when the number of *S. guianensis* accessions was increased to 150, the number of alleles per locus was equal to the variation (2 to 11) and average (4.7) observed here for *S. macrocephala* (Santos et al. unpublished data). The allele's sizes agreed with the expected sizes of Santos et al. (2009c), except for few differences when a higher number of alleles were observed for the same loci.

Individual markers detected from 0.02 to 0.85 of expected heterozygosity, with an average of 0.36. The observed heterozygosity was low, ranging from 0.01 to 0.17 and averaging 0.08 across all loci. Because we used bulked samples, observed heterozygosity could be explained by outcrossing and heterozygous individuals or by heterogeneity in the genbank accessions (Blair et al. 2009).

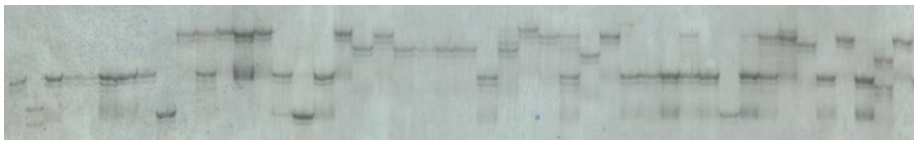

Figure 1 – Pattern of the loci SM02-G5 in 46 accessions of *S. macrocephala*.

Table 2 – The 13 microsatellite loci used to analyse the Embrapa-Cerrados germplasm collection of *S. macrocephala*, with respective number of alleles (N) and observed and expected heterozygosities (H_O and H_E, respectively).

Loci	N	H_O	H_E
SM02 A5	2	0.008	0.07
SM02 A10	3	0.008	0.02
SM01 D3	2	0.02	0.04
SM02 A2	11	0.19	0.85
SM01 B11	7	0.05	0.70
SM02 C9	3	0.04	0.07
SM02 G2	2	0.07	0.26
SM02 G5	8	0.14	0.80
SM01 B5	2	0.02	0.04
SM01 B6	3	0.07	0.36
SM02 A8	3	0.16	0.54
SM02 A9	4	0.08	0.14
SM02 G3B	11	0.17	0.75

Genetic distances among *S. macrocephala* accessions ranged from 0 to 0.83 and averaged 0.54. Barros et al. (2005) studied a subset of 86 accessions from this same *S. macrocephala* germplasm collection using 15 RAPD primers and reported genetic distances from 0.02 to 0.42. Hence, the microsatellite markers were capable of detecting more variation than RAPD's, as it has been shown in studies of other species (Powell et al. 1996, Sun et al. 1999 , Laborda et al. 2005). The genetic distances here described for *S. macrocephala* were higher than the ones reported for other *Stylosanthes* species. In *S. scabra*, genetic dissimilarity was 0.06 among accessions from Brazil, Colombia and Venezuela. In *S. guianensis,* genetic distances averaged 0.26 among 31 accessions assessed by RAPD markers (Kazan et al. 1993). As for AFLP

analysis, the genetic distances among 42 *S. guianensis* accessions varied from 0.05 to 0.69 (Chang-Shun et al. 2004). Besides the more polymorphic nature of microsatellites, the higher genetic distances estimated here can also be due to the higher number of accessions studied. A recent analysis of 150 *S. guianensis* accessions using 20 microsatellite markers resulted in high genetic distance values as well (Santos et al. unpublished data). Although RAPD showed a smaller degree of genetic distances for *S. macrocephala*, we were not able to discriminate all accessions with many possible replicates being identified, whereas no replicates were described in the study of Barros et al. (2005).

The population structure in the 134 *S. macrocephala* accessions was assessed using STRUCTURE 2.0. It comprises a Bayesian clustering approach that probabilistically assigns individuals to populations based on their genotypes. This analysis identified 5 groups in the germplasm collection of *S. macrocephala* (Fig. 2A). Using K=5, 57% of the accessions were assigned to one group with more than 80% probability. The remaining 59 accessions were considered as mixed ancestry. Groups C and E were the ones with the highest numbers of admixture accessions, respectively with 59% and 62% of the accessions showing less than 80% membership. Group D comprised the lowest number of accessions with admixture being 79% of individuals assigned with more than 80% probability. When those groups were considered as different populations, the observed heterozygosity varied from 0.03, in group D, to 0.14, in group C, while the expected heterozygosity ranged from 0.14, in group D, to 0.38, in group C. Hence, group D was the most homogeneous with

the lowest genetic diversity whereas group C showed the highest genetic diversity.

The proportion of genetic diversity due to differences among the *S. macrocephala* groups clustered by STRUCTURE was assessed by the estimate of Nei's gene diversity among groups (G_{ST}). It indicated that approximately 27% of the diversity was due to differences among groups, which is similar to the observed for populations of species belonging to Fabaceae family using isozymes (Hamrick and Godt 1996). In *S. humilis* accessions from Mexico and South America, AFLP studies estimated 30% of variation between the accessions from these two regions (Vander Stappen et al. 2000). Another analysis of *S. humilis* based on AFLP estimated 59% of variation among groups while in *S. viscosa* the estimated variation among groups was 66%, thus identifying a higher degree of genetic differentiation (Sawkins et al. 2001).

As shown in Table 1, the accessions represented the Brazilian States of Minas Gerais, Goias, Bahia and Distrito Federal, with one accession sampled in the State of Piauí and several of unknown origin. Group A (Fig. 2A) comprised accessions from Bahia and Goias. Groups B and E included accessions from Bahia and Minas Gerais. Group C consisted mostly of accessions from Bahia, while group D included accessions from Bahia, Goiás and the Distrito Federal. Barros et al. (2005) described 10 groups of *S. macrocephala* inferred from RAPD, and 75% of all accessions were clustered in only one group while seven of the remaining groups consisted of no more than two accessions. This clustering of 75% of the accessions in a same group limited the analysis of the genetic diversity in the *S. macrocephala* germplasm collection. Besides it

restricts the comparison between those RAPD-derived clusters and the clusters inferred from microsatellites. In the present work, the Bayesian approach enabled the identification of genetic variation patterns among five *S. macrocephala* clusters and clarified the relationships among accessions within the same RAPD cluster previously described by Barros et al. (2005).

Based on STRUCTURE cluster analysis and heterozygosity values, it could be hypothesized that the *S. macrocephala* populations were derived from the population from the state of Bahia and are still in differentiation process. Accessions from Bahia were distributed in all five groups, indicating that those accessions are ancestors to the accessions from other states. Besides, group C, which is major formed by accessions from Bahia, presented the higher expected heterozygosity value, which also indicates a center of origin in this state. This hypothesis deserves further investigation, including natural population analysis.

A tree was constructed based on the Roger's genetic distances and Neighbor-Joining (NJ) clustering method (Fig. 2B). In general, the groups formed by STRUCTURE were subdivided in more than one group in the distance method. In the NJ analysis, group C, which was the most diverse of the groups was subdivided into four groups while groups A and D remained mostly together. Groups B and E were mostly divided in two distinct groups. The Neighbor- Joining tree and STRUCTURE are approaches with different assumptions and differences can be observed when comparing their results (Wang et al. 2009). Model-based approaches, such as implemented in STRUCTURE have shown to be more efficient than distance methods for

descriminating genetic groups, since the cluster identification does not suffer influence from the genetic distance and the graphical representation chosen (Pritchard et al. 2000). Concurrently, both analyses can lead to a deeper understanding of the genetic diversity pattern in the *S. macrocephala* germplasm collection.Genetic diversity is the basis for genetic improvement and knowledge about germplasm diversity has a significant impact on plant breeding (Huang et al. 2002).

The present microsatellite analysis of *S. macrocephala* demonstrated a relatively high genetic diversity and identified population structure among accessions of the Embrapa-Cerrados germplasm collection. Furthermore, the data indicated that microsatellite markers are effective for the assessment of genetic diversity in the *S. macrocephala* species.

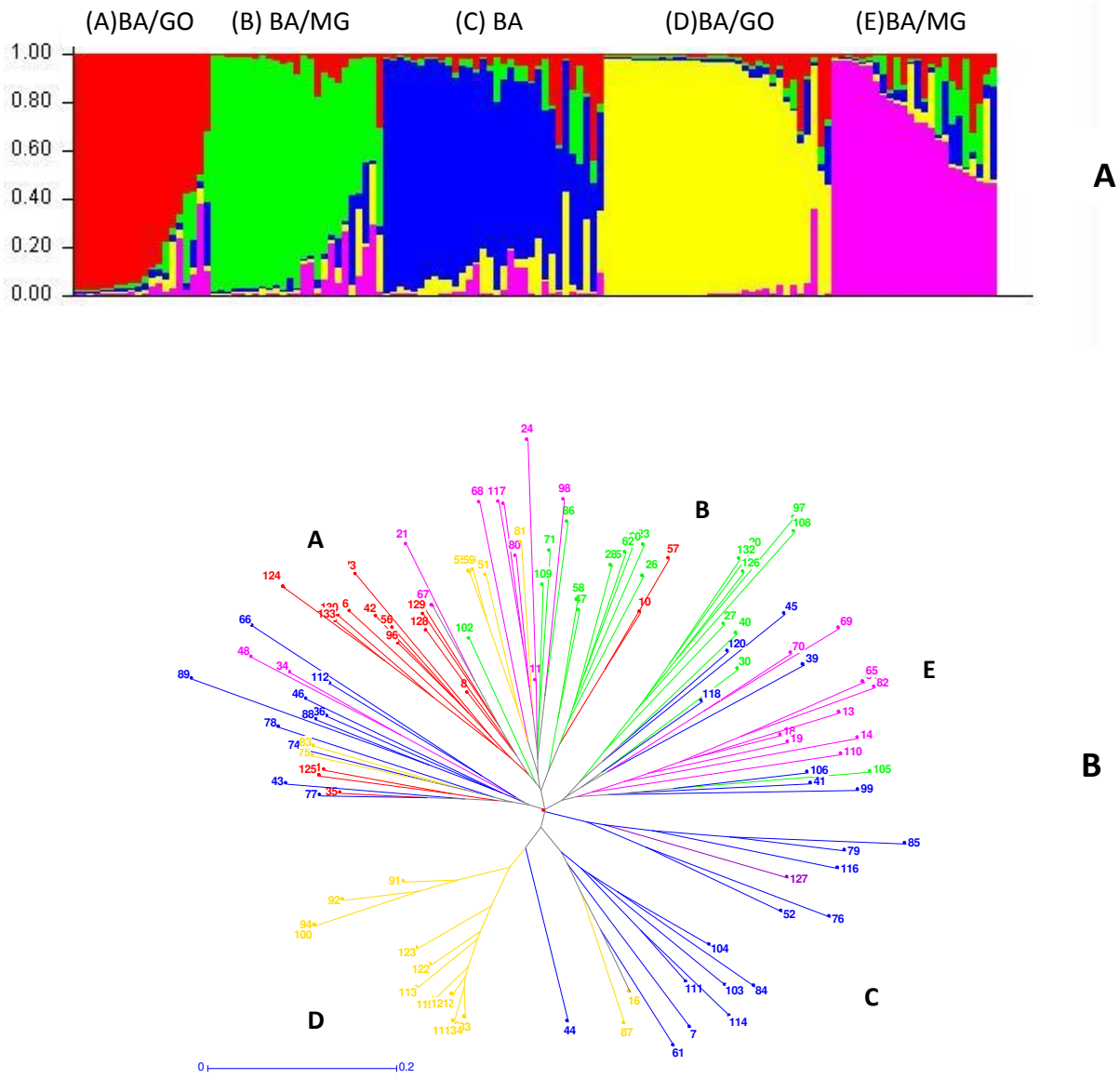

Figure 2 – A - Barplot obtained from the model-based analysis of ancestry of 134 accessions of *S. macrocephala*, implemented in the software Structure. B - Tree derived from the genetic distance of Roger's modified by Wright of 134 accessions of *S. macrocephala*, using the Neighbor- Joining method implemented in the software DARwin. Collors in the DARwin tree represents the color of the groups in the barplot.

References

Andrade RP, Karia CT, Ramos AK. 2004. *Stylosanthes* as a forage legume as its centre of diversity. In: Chakraborty, S, High-yielding anthracnose-resistant *Stylosanthes* for agricultural systems. Canberra: CSIRO, 39-50.

Barros AM, Faleiro FG, Karia CT, Shiratsuchi LS, Andrade RP, Lopes GK. 2005. Variabilidade genética e ecológica de *Stylosanthes macrocephala* determinadas por RAPD e SIG. Pesquisa Agropecuária Brasileira 40: 899-909.

Blair M, Diaz J, Hidalgo R, Diaz L, Duque M. 2007. Microsatellite characterization of Andean races of common bean (*Phaseolus vulgari*s L.). Theoretical and Applied Genetics 116: 29-43.

Blair MW, Díaz LM, Buendía HF, Duque MC. 2009. Genetic diversity, seed size associations and population structure of a core collection of common beans (*Phaseolus vulgaris* L.). Theoretical and applied genetics. Online first, doi: 10.1007/s00122-009-1064-8.

Chang-Shun J, Hu-Sen J, Xin-Rong M, Dong-Mei Z, Yi-Zheng Z. 2004. AFLP analysis of genetic variability among *Stylosanthes guianensis* acessions resitant and susceptible to stylo anthracnose. Acta Botanica Sinica 46: 480-488.

Costa N. 2006. Revisão do género *Stylosanthes*. PhD thesis. Universidade Técnica de Lisboa : 470p.

Costa NM, Ferreira MB. 1984. Some Brazilian species of *Stylosanthes*. In: Stace, HM, Edye, L, The biology and agronomy of *Stylosanthes*. Sydney: Academic Press, 23-48.

Creste S, Tulmann Neto A, Figueira A. 2001. Detection of single sequence repeat polymorphisms in denaturing polyacrylamide sequencing gels by silver staining. Plant Molecular Biology Reporter 19: 299-306.

Edye L, Cameron D. 1984. Prospects for *Stylosanthes* improvement and utilization. In: Stace, H, Edye, L, The biology and agronomy of *Stylosanthes*. Sydney: Academic Press, 571-588.

Evanno G, Regnaut S, Goudet J. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular ecology 14: 2611-20.

Faleiro FG, Faleiro AS, Cordeiro MR, Karia CT. 2003. Metodologia para operacionalizar a extração de DNA de espécies nativas do cerrado visando a análises moleculares. Comunicado Técnico Embrapa Cerrados 92: 5.

Ferreira M, Costa N. 1979. O genero *Stylosanthes* Sw. no Brasil. Belo Horizonte: EPAMIG.

Ferriol M, Pico B, de Cordova P, Nuez F. 2004. Molecular diversity of a germplasm collection of squash (*Cucurbita moschata*) determined by SRAP and AFLP markers. Crop Science 44: 653–664.

Gillies AC, Abbott RJ. 1996. Phylogenetic relationships in the genus *Stylosanthes* (Leguminosae) based upon chloroplast DNA variation. Plant Systematics and Evolution 200: 1615-6110.

Goudet J. 2001. FSTAT (Version 1.2): a computer program to calculate F-statistics. Available at: http://www2.unil.ch/popgen/softwares/fstat.htm.

Hamrick J, Godt M. 1996. Effects of life history traits on genetic diversity in plant species. Philosophical Transaction of the Royal Society B 351: 1291-1298.

Hao CY, Zhang XY, Wang LF, Dong YS, Shang XW, Jia JZ. 2006. Genetic Diversity and Core Collection Evaluations in Common Wheat Germplasm from the Northwestern Spring Wheat Region in China. Molecular Breeding 17: 69-77.

Huang Q, Börner a, Röder S, Ganal W. 2002. Assessing genetic diversity of wheat (*Triticum aestivum* L.) germplasm using microsatellite markers. Theoretical and applied genetics 105: 699-707.

Karia CT. 2008. Caracterização genética e morfoagronomica de germoplasma de *Stylosanthes guianensis* (Aubl.) Sw. PhD Thesis. Universidade Federal de Goiás: 140p.

Kazan K, Manners JM, Cameron DF. 1993. Genetic relationships and variation in the *Stylosanthes guianensis* species complex assessed by random amplified polymorphic DNA. Genome 36: 43-49.

Kelemu S, Changshun J, Guixi H, Segura G. 2005. Genetic transformation of the tropical forage legume *Stylosanthes guianensis* with rice-chitinase gene confers resistance to *Rhizoctonia* foliar blight disease. African Journal of Biotechnology 4: 1025-1033.

Laborda PR, Oliveira KM, Garcia AA, Paterniani ME, Souza AP. 2005. Tropical maize germplasm: what can we say about its genetic diversity in the light of molecular markers? Theoretical and Applied Genetics 111: 1288-1299.

Landjeva S, Korzun V, Ganeva G. 2006. Evaluation of Genetic Diversity Among Bulgarian Winter Wheat (*Triticum aestivum* L.) Varieties During the Period

1925–2003 Using Microsatellites. Genetic Resources and Crop Evolution 53: 1605-1614.

Lewis PO, Zaykin D. 2000. Genetic Data Analysis: Computer program for the analysis of allelic data. Available at: http://alleyn.eeb.uconn.edu/gda/ 2000.

Liu C, Musial J, Thomas B. 1999. Genetic relationship among *Stylosanthes* species revealed by RFLP and STS analyses. Theoretical and Applied Genetics 99: 1179-1186.

Miller MP. 1997. Tools for population genetic analysis (TFPGA): A Windows program for the analysis of allozyme and molecular population genetic data. Available at: http://www.marksgeneticsoftware.net/tfpga.htm.

Pathak P, Ramesh C, Bhatt R. 2004. *Stylosanthes* in the reclamation and development of degraded soils in India. In: S, C, High-yielding anthracnose-resistant *Stylosanthes* for agricultural systems. Canberra: Australian Centre for International Agricultural Research, 85-96.

Perrier X, Jacquemoud-Collet J. 2006. DARwin software. Available at: http://darwin.cirad.fr/darwin.

Phaikaew C, Ramesh C, Kexian Y, Stur W. 2004. Utilisation of *Stylosanthes* as a forage crop in Asia. In: Chakraborty, S, High-yielding anthracnose-resistant Stylosanthes for agricultural systems. Canberra: CSIRO, 65-76.

Powell W, Morgante M, Andre C, Hanafey M, Vogel J, Tingey S, Rafalski A. 1996. The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. Molecular Breeding 2: 1572-9788.

Pritchard JK, Stephens M, Donnelly P. 2000. Inference of Population Structure Using Multilocus Genotype Data. Genetics 155: 945-959.

Santos MO, Sassaki RP, Chiari L, Resende RM, De Souza AP. 2009a. Isolation and characterization of microsatellite loci in tropical forage *Stylosanthes capitata* Vogel. Molecular Ecology Resources 9: 192-194.

Santos MO, Karia CT, Resende RM, Chiari L, Jungmann L, Zucchi MI, Souza AP. 2009b. Isolation and characterization of microsatellite loci in the tropical forage legume *Stylosanthes guianensis* (Aubl.) Sw. Conservation Genetics Resources: 2-5.

Santos MO, Sassaki RP, Ferreira THS, Resende RMS, Chiari L, Karia CT, Faleiro FG, Jungmann L, Zucchi MI, Souza AP. Polymorphic microsatellite loci for *Stylosanthes macrocephala* Ferr. et Costa, a tropical forage legume. 2009c. Conservation Genetics Resources. Online first, DOI 10.1007/s12686-009-9112-x.

Sawkins MC, Maass BL, Pengelly C, Newburry HJ, Ford-Lloyd BV, Maxted N, Smith R. 2001. Geographical patterns of genetic variation in two species of *Stylosanthes* Sw. using amplified fragment lenght polymorphism. Molecular Ecology 11: 1947-1958.

Stace H, Cameron D. 1984. Cytogenetics and the evolution of *Stylosanthes*. In: Stace, H, Edye, L, The biology and agronomy of *Stylosanthes*. Sydney: Academic Press, 49-72.

Struss D, Plieske J. 1998. The use of microsatellite markers for detection of genetic diversity in barley populations. Theoretical and Applied Genetics 97: 308-315.

Sun G, Díaz O, Salomon B, Von Bothmer R. 1999. Genetic diversity in *Elymus caninus* as revealed by isozyme, RAPD, and microsatellite markers. Genome 42: 420–431.

t' Manetje L. 1984. Considerations on the taxonomy of the genus *Stylosanthes*. In: Stace, H, Edye, L, The biology and agronomy of *Stylosanthes*. Sydney: Academic Press, 1-22.

Vander Stappen J, Weltjens I, Lopez S, Volckaert G. 2000. Genetic diversity in Mexican *Stylosanthes humilis* as revealed by AFLP, compared to the variabiliy of *S. humilis* accessions of South American origin. Euphytica 113: 145-154.

Wang ML, Zhu C, Barkley Na, Chen Z, Erpelding JE, Murray SC, Tuinstra MR, Tesso T, Pederson Ga, Yu J. 2009. Genetic diversity and population structure analysis of accessions in the US historic sweet sorghum collection. TAG. Theoretical and applied genetics. Online first, doi: 10.1007/s00122-009-1155-6.

Westman AL, Kresovich S. 1997. Use of molecular marker techniques for description of plant genetic variation. . In: Callow, JA, Ford-Lloyd, BV, Newbury, JH, Plant Genetic resources. Conservation and Use. Wallingford: CAB International, 9-48.

Williams RJ, Reid R, Schultze-Kraft R, Costa NM, Thomas BD. 1984. Natural distribution of *Stylosanthes*. In: Stace, H, Edye, L, The biology and agronomy of *Stylosanthes*. Sydney: Academic Press, 73-101.

Wright S. 1978. Evolution and the genetics of populations, vol.4. Variability within and among natural populations. Chicago: University of Chicago Press. 590p.

Zong X, Redden RJ, Liu Q, Wang S, Guan J, Liu J, Xu Y, Liu X, Gu J, Yan L, Ades P, Ford R. 2009. Analysis of a diverse global *Pisum* sp. collection and comparison to a Chinese local *P. sativum* collection with microsatellite markers. Theoretical and applied genetics. 118: 193-204.

8 Artigo V

"Isolation and charactherization of microsatellite loci in the tropical forage legume *Stylosanthes guianensis* (Aubl.) Sw."

Publicado na Conservation Genetics Resources (Online first, DOI 10.1007/s12686-009-9010-2)

Autores: Melissa de O. Santos, Cláudio T. Karia, Rosângela M. S. Resende, Lucimara Chiari, Letícia Jungmann, Maria I. Zucchi, Anete P. Souza.

Conservation Genet Resour
DOI 10.1007/s12686-009-9010-2

TECHNICAL NOTE

Isolation and characterization of microsatellite loci in the tropical forage legume *Stylosanthes guianensis* (Aubl.) Sw.

M. O. Santos · C. T. Karia · R. M. S. Resende · L. Chiari · L. Jungmann · M. I. Zucchi · A. P. Souza

Received: 16 April 2009 / Accepted: 18 April 2009

Abstract *Stylosanthes guianensis* is an important tropical pasture legume. Knowledge of genetic diversity and structure of *S. guianensis* populations is of great importance for the conservation and germplasm management of this species. Thus, 20 microsatellite markers were developed from a *S. guianensis* enriched library. The microsatellites were characterized in 20 accessions from the germplasm collection of the Empresa Brasileira de Pesquisa Agropecuária (Embrapa). The average number of alleles per locus varied from 2 to 7, with an average of 4 alleles per locus. The observed and expected heterozygosities ranged from 0 to 0.60 and 0.10 to 0.85, respectively. This new set of microsatellites will contribute towards studies of genetic diversity and conservation of *S. guianensis*.

Keywords Molecular markers · Genetic diversity · Conservation · Genomic enriched library

M. O. Santos · A. P. Souza (✉)
Centro de Biologia Molecular e Engenharia Genética, Departamento de Genética e Evolução, Universidade Estadual de Campinas, Barão Geraldo, CP 6010, Campinas, SP CEP 13083-970, Brazil
e-mail: anete@unicamp.br

C. T. Karia
Empresa Brasileira de Pesquisa Agropecuária (Embrapa)—Cerrados, BR 020, Km 18, Planaltina, DF CEP 73310-970, Brazil

R. M. S. Resende · L. Chiari · L. Jungmann
Empresa Brasileira de Pesquisa Agropecuária (Embrapa)—Gado de Corte, BR 262, Km 4, Campo Grande, MS CEP 79002-970, Brazil

M. I. Zucchi
Instituto Agronômico de Campinas (IAC), Av. Barão de Itapura n° 1481, CP 28, Campinas, SP CEP 13012-970, Brazil

A. P. Souza
Instituto de Biologia, Departamento de Biologia Vegetal, Universidade Estadual de Campinas, Barão Geraldo, CP 6109, Campinas, SP CEP 13083-970, Brazil

Published online: 05 May 2009

The genus *Stylosanthes* Sw. (Fabaceae) comprises about 40 species distributed in the tropical areas of America, Africa and Southeast Asia (Costa and Ferreira 1984; Mannetje 1984; Williams et al. 1984). Brazil is the major center of origin and diversity of *Stylosanthes*, where 25 out of the 40 species of this genus are found (Ferreira and Costa 1979; Andrade and Karia 2000). *Stylosanthes* species have high protein content and good development in relatively poor soil. They are used for soil improvement through nitrogen fixation, reclaiming degraded wastelands, and for water and soil conservation as well (Baldión et al. 1975; Kelemu et al. 2005). Moreover, some of the *Stylosanthes* species are considered the most important source of pasture legume for tropical and subtropical environments (Edye and Cameron 1984).

Stylosanthes guianensis is a diploid species that belongs to the Stylosanthes section (Mannetje 1984). It displays great morphological diversity, being the most widespread among *Stylosanthes* species (Williams et al. 1984). *Stylosanthes guianensis* is native to South and Central America, with an extensive distribution in those areas except for the equatorial zone where it is scarcely recorded (Williams et al. 1984). The isolation and characterization of novel microsatellites from a genomic library of *Stylosanthes guianensis* are reported herewith and they were demonstrated to be potentially useful for further genetic studies of this species.

Genomic DNA was isolated from *S. guianensis* fresh leaves using the cetyltrimethyl ammonium bromide (CTAB)

method (Doyle and Doyle 1990) with modifications made for the genus by Bonato et al. (2002). A genomic enriched library was constructed following the protocol described by Billotte et al. (1999). The purified total DNA was digested with *Rsa* I and enriched in $(CT)_8$ and $(GT)_8$ repeats. Enriched fragments were amplified by polymerase chain reaction (PCR), ligated into a pGem-T Easy vector (Promega) and then transformed into competent XL1-blue *Escherichia coli*

Table 1 Characteristics of 20 microsatellite loci from *Stylosanthes guianensis*

Locus	GenBank acession number	Primer sequences (5′–3′)	Repeat motif	N	Size range (bp)	H_o	H_e	*P* value HWE
SG03B5	FJ820272	5′AGGTGGATGCGAGTTCTT3′ 5′TCCCTTCTACCGAGTGTTC3′	(TG)7	2	180–200	0	0.10	0.000*
SG03D4	FJ820273	5′GATCGGTCGGGTTGGCTACTAT3′ 5′CCTATTATCCCCATTCCTCACA3′	(GT)8	3	250–260	0	0.56	0.000*
SG03E1	FJ820274	5′CCAAAGCGTAGAGAATGATGAG3′ 5′CAAATGGAGCGAAAGGACAA3′	(GT)8	2	266–268	0	0.36	0.000*
SG03D1	FJ820275	5′GTGGCGAAAAATCTAAAATGTC3′ 5′GGTGGAATCCCTAACTGAAGA3′	(GT)6	4	174–180	0.05	0.60	0.000*
SG03G2	FJ820276	5′GGTTGGAATATGGAGGAAGA3′ 5′GAGGAAAACTAAACAAAGCAGA3′	(GT)6	5	200–220	0.05	0.72	0.000*
SG03G4	FJ820277	5′TTGCCTTTATCCTTGTCACTCA3′ 5′ATCAAGAATCCAATACCAAATG3′	(TG)8	4	214–222	0.10	0.68	0.000*
SG01B9	FJ820278	5′TTACGCGAAAACCCGAACA3′ 5′GCACCTACAAAAGCTACACCAT3′	(AC)7	5	300–330	0.55	0.71	0.662
SG03G8	FJ820279	5′AATTAAAGAGGAGGAGGAAAGT3′ 5′TGGAGAAGTAAAAGACAGTGAG3′	(CT)11…(CA)9	6	196–226	0.06	0.81	0.000*
SG01C2	FJ820280	5′TGAGAAGCACAAGGGATAAGGA3′ 5′CGAACCGGACCAAACCAT3′	(GT)7	2	308–310	0	0.29	0.000*
SG01B12	FJ820281	5′ATTGTTAGGGATAGGTGATTA3′ 5′TCGGAGTTGATTTGATTATG3′	(AC)7…(CT)4(TC)4	2	290–292	0.18	0.40	0.024
SG01D7	FJ820282	5′ACATAAACTGGACAGGGTGATT3′ 5′ATTTTTGCGAGGTGCTAAGTC3′	(AC)7	3	196–222	0.60	0.62	0.796
SG01D3	FJ820283	5′AGATGGGCTAGATACGGAGATA3′ 5′TCGGAGTTGGTTTGATGGT3′	(AC)6…(AC)7	2	280–292	0.19	0.18	0.679
SG03A9	FJ820284	5′AGTCCCAGTACCCAGAACA3′ 5′AACCCCTTTTTAACACAACA3′	(CA)7	2	198–200	0	0.34	0.002*
SG03E10	FJ820285	5′TCCCAGCTCGTATGAAGAAGTT3′ 5′GGACCCGGAGCACCTATC3′	(GT)7	7	218–238	0.21	0.85	0.014
SG03B10	FJ820286	5′CTCTAAACGATGAAAATGAACG3′ 5′AAAGGAACAAAGGACGAGACAG3′	(GT)3G(GT)4 (TG)3(GT)6	7	292–310	0.21	0.82	0.000*
SG03E2	FJ820287	5′GTGCCCTTGAGCCCCCTTTAGT3′ 5′GAGCGGCGATCGGAGTTTGT3′	(AC)8	7	152–210	0.29	0.73	0.001*
SG03E7	FJ820288	5′AAGATGGGCCAAAAGGAACAAA3′ 5′TGGAGTGGCTTACCGTGATTAC3′	(AC)6…(CT)5	4	282–292	0.07	0.73	0.000*
SG03A7	FJ820289	5′TACGGAAGTCCCATTAGTGAGG3′ 5′GGCTGCCGGAAGTTGACG3′	(AG)8	5	206–232	0.17	0.72	0.001*
SG03E9	FJ820290	5′GGTCAAATGGGGCAAAAGA3′ 5′ATCGAAGAGGAAAAGGCTAACT3′	(AC)5…(CA)5	2	238–242	0.21	0.39	0.109
SG01A7	FJ820291	5′TACCTTGAATCCGCACCTATGA3′ 5′CACCCGAACACCTAATCCTAAA3′	(AG)5…(GT)8	4	300–330	0.33	0.46	0.058

Shown are the loci names, the GenBank accession number, the forward (F) and reverse (R) primer sequence, repeat motif, number of alleles (N), product size range in base pairs, observed (H_o) and expected (H_e) heterozygosities and *P* value HWE

* Departs significantly from HWE at $P < 0.05$ after Bonferroni correction

Table 2 Cross—species amplification of 20 microsatellite loci from *Stylosanthes guianensis* in *Stylosanthes capitata*, *S. macrocephala* and *S. pilosa*

Locus	*S. capitata*	*S. macrocephala*	*S. pilosa*
SG03B5	−	−	−
SG03D4	+	+	+
SG03E1	−	−	−
SG03D1	+	+	+
SG03G2	+	+	+
SG03G4	+	+	+
SG01B9	−	+	−
SG03G8	+	+	+
SG01C2	−	+	−
SG01B12	−	−	−
SG01D7	+	+	+
SG01D3	−	−	−
SG03A9	−	−	−
SG03E10	−	−	−
SG03B10	−	−	−
SG03E2	−	−	−
SG03E7	+	+	+
SG03A7	−	−	−
SG03E9	−	−	−
SG01A7	−	+	−

Successful amplification (+) and failed amplification (−) are indicated

cells. The positive clones were selected using the β-galactosidase gene and grown overnight in an HM/FM medium with ampicillin. After PCR, 144 positive clones were sequenced in both directions using the T7 and SP6 primers as well as the v3.1 Big Dye terminator kit (PerkinElmer Applied Biosystems) with an ABI PRISM® 377. The sequences were assembled and edited in Seqman (DNAStar). The repetitive regions were identified using the Simple Sequence Repeat Identification Tool (Temnykh et al. 2001). Forty-six of these clones contained microsatellite sequences with more than five repeats and flanking regions suitable for primer design. The software Primer Select (DNAStar) was used to design 46 primer pairs complementary to the flanking regions of those repetitive regions.

Polymorphism was tested and characterized for the isolated microsatellite loci using 20 accessions of the *S. guianensis* of the Empresa Brasileira de Pesquisa Agropecuária (Embrapa) Cerrados. DNA samples were isolated from bulks of four plants using the CTAB method (Doyle and Doyle 1990) with few modifications (Bellon et al. 2007). The PCR amplifications were performed using a PTC-200 (MJ Research) thermocycler in a total reaction volume of 15 µl, consisting of 1X PCR buffer, 1.5 mM $MgCl_2$, 0.25 mM of each dNTP (Invitrogen), 0.8 µM of each primer, 1U *Taq* DNA polymerase (Invitrogen) and 10 ng of genomic DNA. The amplification protocol consisted of an initial denaturation at 94°C for 1 min, 30 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min and a final extension at 72° for 5 min. The amplification products were separated in 6% denaturing polyacrylamide gels running at 75 W for about 120 min. The polyacrylamide gels were silver stained (Creste et al. 2001) and the allele scoring was done using the 10 pb Ladder (Invitrogen).

Of the 46 developed microsatellites, 20 were polymorphic, with the number of alleles per locus ranging from 2 to 7 and average of 4 alleles per locus. Descriptive statistics and the test for Hardy–Weinberg Equilibrium were performed using Tools for Genetic Population Analysis (TFPGA) (Miller 1997). The observed and expected heterozygosities ranged from 0 to 0.60 (0.16 on average) and 0.10 to 0.85 (0.55 on average), respectively. Thirteen loci depart significantly from Hardy–Weinberg Equilibrium ($P < 0.05$) after Bonferroni correction and the low observed heterozygosity values may be due to the predominance of autogamy in this species (Table 1). The linkage disequilibrium was tested using the Popgene 1.32 (Yeh et al. 1998) and no disequilibrium was detected among all loci. Cross-species amplification was evaluated on *Stylosanthes macrocephala*, *S. capitata* and *S. pilosa*. Seven of the 20 evaluated loci were amplified in all these species and three other loci amplified only in *S. macrocephala* (Table 2).

The microsatellites developed in this work are potentially useful as a tool in studies of the genetic diversity of the *S. guianensis* germplasm collections and the genetic structure of natural populations.

Acknowledgments The authors would like to thank Fundação de Amparo à Pesquisa do Estado de São Paulo for the financial support during the development of this research (2005/51010-0). Santos MO received a PhD scholarship from FAPESP (2005/52211-9). Souza AP is recipient of a research fellowship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

References

Andrade RP, Karia CT (2000) O uso de Stylosanthes em pastagens no Brasil. In: SIMPÓSIO DE FORRAGICULTURA E PASTAGEM: TEMAS EM EVIDÊNCIA, 1. 2000. Lavras. Anais… Lavras: UFLA, 273–309

Baldión R, Lozano JC, Grof B (1975) Evaluación de la resistência de *Stylosanthes* spp. a la antracnosis (*Colletotrichum gloeosporioides*). Fitopatologia 10:104–108

Bellon G, Faleiro FG, Ferreira CF, Karia CT, Fonseca KG, Santos JRP, Teixeira MA, Junqueira KP (2007) Validação e otimização de protocolo simplificado para extração de DNA a partir de tecido foliar. In: CONGRESSO BRASILEIRO DE MELHORAMENTO DE PLANTAS, 4., 2007, São Lourenço. Anais… Lavras: UFLA, Sociedade Brasileira de Melhoramento de Plantas

Billotte N, Lagoda PJR, Risterucci AM, Baurens FC (1999) Microsatellite-enriched libraries: applied methodology for the development of SSR markers in tropical crops. Fruits 54:277–288

Bonato ALV, Verzignassi JR, Resende RMS, Fernandes CD, Leguizamón GOC (2002) Extração de DNA genômico de *Stylosanthes* spp. Comunicado Técnico 78. Empresa Brasileira de Pesquisa Agropecuária, EMBRAPA

Costa NMS, Ferreira MB (1984) Some Brazilian species of *Stylosanthes*. In: Stacce HM, Edye LA (eds) The biology and agronomy of *Stylosanthes*. Academic Press, Sydney, Australia, pp 23–48

Creste S, Tulmann Neto A, Figueira A (2001) Detection of single sequence repeat polymorphisms in denaturing polyacrylamide sequencing gels by silver staining. Plant Mol Biol Rep 19:299–306

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12:13–15

Edye LA, Cameron DG (1984) Prospects for *Stylosanthes* improvement and utilization. In: Stace HM, Edye LA (eds) The biology and agronomy of *Stylosanthes*. Academic Press, Sydney, Australia, pp 571–587

Ferreira MB, Costa NMS (1979) O gênero *Stylosanthes* Sw. No Brasil. EPAMIG, Belo Horizonte

Kelemu S, Changshun J, Guixi H, Segura G (2005) Genetic transformation of the tropical forage legume *Stylosanthes guianensis* with a rice-chitinase gene confers resistance to *Rhizoctonia* foliar blight disease. Afr J Biotechnol 10:1025–1033

Mannetje Lt' (1984) Considerations on the taxonomy of the genus *Stylosanthes*. In: Stace HM, Edye LA (eds) The biology and agronomy of *Stylosanthes*. Academic Press, Sydney, Australia, pp 1–21

Miller MP (1997) Tools for population genetic analysis (TFPGA) 1.3. A windows program for the analysis of allozyme and molecular population genetic data. Computer software distributed by author

Temnykh S, DeClerck G, Lukashova A, Lipovich L, Catinhour S, McCouch S (2001) Computational and experimental analysis of microsatellites in rice (*Oryza sativa* L.): frequency, length variation, transposon associations, and genetic marker potential. Genome 11:1441–1452

Williams RJ, Reid R, Shultze-Kraft R, Costa NMS, Thomas BD (1984) Natural distribution of Stylosanthes. In: Stacce HM, Edye LA (eds) The biology and agronomy of Stylosanthes. Academic Press, Sydney, Australia, pp 73–101

Yeh FC, Rong-Cai Y, Boyle T (1998) POPGENE version 1.31. University of Alberta, Center for International Forestry Research, Edmonton, Alberta, Canada

9 Artigo VI

"Tropical Forage Legumes: III Genetic Diversity in *Stylosanthes guianensis* (Aubl.) Sw."

A ser submetido à revista Theoretical and Applied Genetics

Autores: Melissa de O. Santos-Garcia, Rosângela M. S. Resende, Lucimara Chiari, Cláudio T. Karia, Maria I. Zucchi, Anete P. Souza.

Tropical Forage Legumes: III Genetic Diversity in *Stylosanthes guianensis* (Aubl.) Sw.

M O Santos-Garcia[1], R M S Resende[2], L Chiari[2], C T Karia[3], M I Zucchi[4], A P Souza[1].

[1] Centro de Biologia Molecular e Engenharia Genética, Universidade Estadual de Campinas, Barão Geraldo, Campinas, SP, Brazil. CP 6010, CEP 13083-970.

[2] Empresa Brasileira de Pesquisa Agropecuária (Embrapa) - Gado de Corte. BR 262, Km 4. Campo Grande, MS, Brazil. CEP 79002-970.

[3] Empresa Brasileira de Pesquisa Agropecuária (Embrapa) – Cerrados. BR 020, Km 18.Planaltina, DF – Brazil. CEP 73310-970

[4] Pólo Apta Centro Sul – Rod. SP 127 km 30, Piracicaba – SP, Brazil, CP 28, CEP 13400-970.

Keywords: outcrossing rate, population structure, autogamy, mixed mating system.

*Corresponding author: Anete Pereira de Souza. Centro de Biologia Molecular e Engenharia Genética. Universidade Estadual de Campinas, Campinas, SP, Brazil. CP 6010. CEP 13083-970.

E-mail: anete@unicamp.br

Phone: 55-19-3521-1132

FAX: 55-19-3521-1089

Abstract

Microsatellite loci were used to study the genetic diversity of a germplasm collection of *Stylosanthes guianensis* belonging to Embrapa. The genetic diversity was surveyed in 150 accessions selected as representative sample of the germplasm collection from Embrapa by using 20 microsatellite markers. The number of alleles per locus varied from two to 11, with an average of 4.7. The observed and expected heterozygosities varied respectively from 0 to 0.58 (mean of 0.18) and from 0.04 to 0.83 (mean of 0.55). The low level of observed heterozygosity was in agreement with the outcrossing rate of 26% reported for this species. A model-based Bayesian approach implemented in the software STRUCTURE was used to assign accessions into clusters and a dendrogram was constructed based on the Roger's genetic distances. A total of nine groups were assembled in STRUCTURE and they are consistent with clusters inferred from the genetic distances and with the taxonomic varieties described for this species. The data demonstrated genetic diversity among and within botanical varieties, which is suitable to be exploited in *S. guianensis* breeding programs.

Introduction

Germplasm collections are an important component of plant breeding programs as they provide plant breeders with sources of useful traits (Cordeiro et al. 2003). The optimization of genetic resource management requires precise identification of the genotypes as well as correct evaluations of the amount and distribution of genetic diversity within a species (Krishna et al. 2004, Alcaraz and Hormaza 2007, Blair et al. 2007) , which is highly influenced by the mating system (Hamrick et al. 1979, Hamrick and Godt 1996, Barrett 2003).

Stylosanthes species are considered one of the most important sources of pasture legume for tropical and subtropical environments (Edye and Cameron 1984). It is also used for soil improvement through nitrogen fixation and for reclaiming degraded wastelands (Pathak et al. 2004, Kelemu et al. 2005). The genus *Stylosanthes* Sw. belongs to the tribe Aeschynomeneae, subtribe Stylosanthinae, in the Fabaceae family, consisting of 48 herbaceous and suffruticose species (Costa 2006). Most of its species are distributed in tropical, subtropical and temperate areas of Americas. The main diversity center of this genus is Central Brazil (Ferreira and Costa 1979, Stace and Cameron 1984), which comprises 45% of all *Stylosanthes* species and where the greatest phenotypic variation and endemism are observed (Costa 2006). Mexico and Caribbean Islands, in Central America, are the second major *Stylosanthes* diversty center (Stace and Cameron 1984).

Stylosanthes guianensis is a diploid species (Stace and Cameron 1984) with great morphological diversity, being the most widespread and one of the

most promising among *Stylosanthes* species. It produces great amounts of dry matter and retains leaves during dry season (Williams et al. 1984, Maass and Sawkins 2004). *S. guianensis* is native to South and Central America, where it is widely distributed except for the equatorial zone (Williams et al. 1984). Taxonomic groups within *S. guianensis* have been differently treated by authors, being classified as varieties within the same species (t' Manetje 1984) or as different species and varieties (Ferreira and Costa 1979, Ferreira et al. 1985, Costa 2006).

Methods based on molecular markers, along with morphoagronomical data and ecological characteristics of the collection geographical site, have proven suitable for evaluating germplasm, organizing genetic resources, identifying putative duplicated accessions, determining how variation is distributed between individuals and constructing smaller collections (Zong et al. 2009, Westman and Kresovich 1997). Microsatellites or simple sequence repeats (SSRs) are codominant, highly polymorphic and widely distributed over the genome, being particularly useful for the characterization of germplasm collections (Zong et al. 2009, Liu et al. 2003, Barkley et al. 2006). Evaluations of germplasm collections using microsatellites can optimize and facilitate breeding process.

Considering those assumptions and the importance of *S. guianensis* as forage for tropical areas, this study aimed to characterize the genetic diversity among *S. guianensis* accessions of the germplasm collection of Brazilian Agricultural Research Corporation (Embrapa) Cerrados using microsatellite markers.

Materials and Methods

A total of 150 accessions from the Brazilian Agricultural Research Corporation (Embrapa) Cerrados were selected according to the groups observed by Karia (2008) as a representative sample of this germplasm collection (Table 1).

DNA samples were extracted from fresh leaves using the cetyltrimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1990) with modifications made by Bellon et al. (2007). Leaves from four plants per accession were bulked for DNA extraction. The PCR reactions and DNA amplification were performed as described using twenty microsatellites as described by Santos et al. (2009). Amplified fragments were evaluated in 6% acrylamide gels and visualized by silver-staining (Creste et al. 2001).

Genetic Data Analysis (GDA) (Lewis and Zaykin 2000) was used to calculate observed and expected heterozygosities. Allele frequencies and Roger's distance modified by Wright (1978) were calculated between all pairs of accessions using Tools for Population Genetic Analysis (TFPGA)(Miller 1997). Accessions were subdivided into genetic clusters using a model based clustering with the software package STRUCTURE (Pritchard et al. 2000). STRUCTURE uses Bayesian approach to identify clusters based on fit to Hardy-Weinberg equilibrium and linkage equilibrium. Ten runs of STRUCTURE were done by setting the number of populations (K) from 1 to 20. For each run, burn-in time was set in 200,000 while replication number was set to 300,000. Identification

of number of distinct clusters (K) was performed following the procedure described by Evanno et al. (2005). Graphical representation of population assignments from the program STRUCTURE was done using the program R v.2.9.1. The software DARwin 5.0 (Perrier and Jacquemoud-Collet 2006) was used to define the genetic relationships among accessions based on Roger's genetic distance and UPGMA (Unweighted Paired Group Method) clustering method. FSTAT (Goudet 2001) was used to calculate Nei's G_{ST} among the groups obtained in STRUCTURE analysis.

Table 1 – Acessions of *S. guianensis* used to access the genetic diversity using 20 microsatellites. Shown are the sample codes and respective number of the accessions in the germplasm collection of Embrapa Cerrados, number in CIAT collection, collection site, botanical variety according to Ferreira and Costa, 1979 and Ferreira et al. 1985.

Code	CPAC	Ciat number	Collection Site[1]	Botanical Variety	Group
1	135	1297	Distrito Federal	*Pauciflora*	3
2	464	n.a.	Tocantins	n.a.	4
3	517	1705	Mato Grosso	n.a.	7
4	648	1950	Colombia	*Guianensis*	7
5	662	136	Colombia	n.a.	7
6	1121	1507	Venezuela	*Pauciflora*	3
7	1132	1890	Venezuela	*Pauciflora*	7
8	1139	1975	Colombia	n.a.	7
9	1144	2034	Bahia	*Pauciflora*	3
10	1153	2315	Bahia	n.a.	3
11	1229	n.a.	Minas Gerais	*Microcephala*	1
12	1230	2950	Minas Gerais	*Guianensis*	2
13	1231	2951	Minas Gerais	n.a.	6
14	1235	n.a.	Goiás	n.a.	5
15	1237	2615	Tocantins	*Canescens*	7
16	1239	2659	Pará	n.a.	7
17	1354	2991	Espirito Santo	*Pauciflora*	3
18	1360	2549	Piaui	*Pauciflora*	3
19	1365	2812	Venezuela	*Pauciflora*	3
20	1368	2742	Minas Gerais	*Pauciflora*	3
21	1369	2529	Bahia	*Pauciflora*	7
22	1371	10107	Goiás	*Microcephala*	3
23	1372	2439	Pernambuco	*Pauciflora*	9
24	1619	2748	Venezuela	n.a.	3
25	2203	2987	Bahia	*Pauciflora*	3
26	2464	10993	Tocantins	*Guianensis*	5
27	2725	2974	Bahia	*Pauciflora*	3
28	2734	2458	Paráiba	*Pauciflora*	3
29	2738	2542	Ceará	*Pauciflora*	3
30	2740	2708	Goiás	*Pauciflora*	5
31	2741	2436	Alagoas	*Pauciflora*	5
32	2761	2727	DistritoFederal	*Pauciflora*	3
33	2771	2992	Espírito Santo	*Pauciflora*	3
34	4142	n.a.	Tocantins	*Guianensis*	7
35	4144	n.a.	Tocantins	*Canescens*	7
36	4157	n.a.	Minas Gerais	*Canescens*	8
37	4172	n.a.	Goiás	*Canescens*	8
38	4173	n.a.	Tocantins	*Canescens*	8
39	4174	n.a.	Maranhão	n.a.	7
40	4188	n.a.	Bahia	*Pauciflora*	3
41	4193	n.a.	Bahia	*Pauciflora*	5
42	4199	n.a.	Bahia	*Pauciflora*	5
43	4227	n.a.	São Paulo	*Canescens*	8
44	4233	n.a.	São Paulo	*Canescens*	8

Table 1 – Continuing.

Code	CPAC	Ciat number	Collection Site[1]	Botanical Variety	Group
45	4237	n.a.	Minas Gerais	*Guianensis*	4
46	4238	n.a.	Rio de Janeiro	*Guianensis*	4
47	4239	n.a.	Minas Gerais	*Guianensis*	7
48	4240	n.a.	Rio de Janeiro	*Guianensis*	7
49	4262	n.a.	Minas Gerais	*Guianensis*	4
50	4264	n.a.	Minas Gerais	*Guianensis*	4
51	4286	n.a.	Goiás	n.a.	8
52	4292	n.a.	Goiás	*Pauciflora*	5
53	4300	n.a.	Minas Gerais	*Guianensis*	4
54	4302	n.a.	Rio de Janeiro	*Microcephala*	7
55	4306	n.a.	Minas Gerais	*Guianensis*	4
56	4308	n.a.	Minas Gerais	*Canescens*	8
57	4314	n.a.	Pará	*Guianensis*	7
58	4315	n.a.	Pará	*Guianensis*	7
59	4322	n.a.	Pará	*Guianensis*	7
60	4323	n.a.	Pará	*Guianensis*	7
61	4324	n.a.	Pará	*Guianensis*	7
62	4331	n.a.	Pará	*Guianensis*	7
63	4336	n.a.	São Paulo	n.a.	8
64	4338	n.a.	São Paulo	n.a.	8
65	4364	n.a.	São Paulo	n.a.	8
66	4528	n.a.	Goiás	n.a.	7
67	4530	n.a.	n.a.	*Guianensis*	2
68	5187	n.a.	Minas Gerais	*Pauciflora*	5
69	5192	n.a.	Minas Gerais	*Pauciflora*	6
70	5204	n.a.	Goiás	*Canescens*	8
71	5206	n.a.	Minas Gerais	*Canescens*	8
72	5221	n.a.	São Paulo	*Canescens*	8
73	5233	n.a.	São Paulo	*Guianensis*	7
74	5236	n.a.	Minas Gerais	*Microcephala*	1
75	5239	n.a.	Goiás	*Microcephala*	9
76	5248	n.a.	Goiás	*Pauciflora*	3
77	5262	n.a.	Mato Grosso do Sul	*Microcephala*	7
78	5277	n.a.	Minas Gerais	*Pauciflora*	6
79	5279	n.a.	Minas Gerais	*Microcephala*	1
80	5294	n.a.	n.a.	n.a.	6
81	5295	n.a.	n.a.	n.a.	9
82	5349	n.a.	Bahia	n.a.	5
83	1361	2445	Pernambuco	n.a.	3
84	5426	n.a.	n.a.	n.a.	5
85	5428	10488	Pará	n.a.	7
86	5429	10416	Pará	n.a.	7
87	5430	2649	Maranhão	n.a.	9
88	5432	2700	Goiás	*Microcephala*	9
89	5433	2677	Tocantins	*Microcephala*	9
90	5435	10124	Minas Gerais	n.a.	4

Table 1 – Continuing.

Code	CPAC	Ciat number	Collection Site[1]	Botanical Variety	Group
91	5436	10126	Espírito Santo	n.a.	4
92	5439	10792	Minas Gerais	*Canescens*	8
93	5440	10793	Minas Gerais	*Canescens*	8
94	5441	10794	Minas Gerais	*Canescens*	8
95	5445	10799	Minas Gerais	*Canescens*	8
96	5447	10802	Minas Gerais	*Canescens*	8
97	5454	10825	Minas Gerais	*Canescens*	8
98	5456	10849	Minas Gerais	n.a.	8
99	5458	10852	Minas Gerais	*Canescens*	8
100	5460	10854	Minas Gerais	*Canescens*	8
101	5462	10876	Minas Gerais	*Canescens*	8
102	5464	10888	Minas Gerais	n.a.	8
103	5469	10808	Minas Gerais	n.a.	1
104	5471	10826	Minas Gerais	*Microcephala*	1
105	5474	10872	Minas Gerais	n.a.	1
106	5476	n.a.	Minas Gerais	*Microcephala*	1
107	5477	10855	n.a.	*Microcephala*	1
108	5484	10814	Minas Gerais	n.a.	8
109	5488	10821	Minas Gerais	*Pauciflora*	3
110	5489	10824	Minas Gerais	*Guianensis*	3
111	5493	10830	Minas Gerais	*Guianensis*	8
112	5500	10838	Minas Gerais	*Guianensis*	4
113	5505	10843	Minas Gerais	*Guianensis*	7
114	5506	10820	Minas Gerais	*Guianensis*	4
115	5509	10847	Minas Gerais	n.a.	4
116	5510	10848	Minas Gerais	*Guianensis*	4
117	5513	10858	Minas Gerais	*Guianensis*	4
118	5514	10859	Minas Gerais	*Guianensis*	4
119	5515	10862	Minas Gerais	*Guianensis*	6
120	5516	10863	Minas Gerais	n.a.	6
121	5518	10866	Minas Gerais	n.a.	9
122	5519	10867	Minas Gerais	n.a.	6
123	5523	10882	Minas Gerais	*Guianensis*	2
124	5525	10886	Minas Gerais	*Guianensis*	4
125	5532	10896	Minas Gerais	n.a.	7
126	5533	10899	Minas Gerais	*Guianensis*	6
127	5536	10904	Minas Gerais	*Guianensis*	5
128	5538	10908	Minas Gerais	n.a.	2
129	EPAMIG-493*	n.a.	n.a.	n.a.	7
130	2745	n.a.	n.a.	n.a.	2
131	EPAMIG-1044*	n.a.	n.a.	n.a.	5
132	EPAMIG-1448*	n.a.	n.a.	n.a.	7
133	EPAMIG-443*	n.a.	n.a.	n.a.	7
134	NC 1099 A*	n.a.	Minas Gerais	n.a.	5
135	NC 2270*	n.a.	Pará	*Guianensis*	
136	10283	n.a.	Minas Gerais	*Guianensis*	

Table 1 – End.

Code	CPAC	Ciat number	Collection Site[1]	Botanical Variety	Group
137	10285	n.a.	Minas Gerais	*Pauciflora*	3
138	IPF 394/75*	n.a.	n.a.	n.a.	7
139	EPAMIG-1529*	n.a.	Minas Gerais	n.a.	3
140	EPAMIG-1557*	n.a.	Minas Gerais	*Guianensis*	4
141	EPAMIG-1670*	n.a.	Minas Gerais	*Guianensis*	5
142	EPAMIG-1787*	n.a.	Minas Gerais	*Guianensis*	2
143	EPAMIG-1691*	n.a.	Minas Gerais	*Guianensis*	7
144	EPAMIG-1994*	n.a.	Minas Gerais	*Guianensis*	2
145	2600	n.a.	Tocantins	n.a.	7
146	2676	n.a.	Tocantins	*Microcephala*	9
147	2689	n.a.	Tocantins	*Microcephala*	9
148	2694	n.a.	Tocantins	*Microcephala*	9
149	LC 4297*	n.a.	n.a.	n.a.	9
150	LC 4471*	n.a.	n.a.	n.a.	3

*Accessions without number in Embrapa Cerrados collection, shown are the identifications in other germplasm collections or the collector number. 1 The collection sites were according to Brazilian states or other countries. n.a. not available.

Results and Discussion

Aiming at studying the amount and the distribution of the genetic diversity in a germplasm collection, we surveyed 150 accessions of the Embrapa Cerrados (Table 1). The accessions were selected according to the clusters described by Karia (2008) in order to represent the genetic diversity of this collection. A limited number of microsatellite loci were available at the time of the germplasm evaluation conducted by Karia (2008). In this present work, the germplasm evaluation was extended to 20 polymorphic microsatellite markers (Fig. 1).

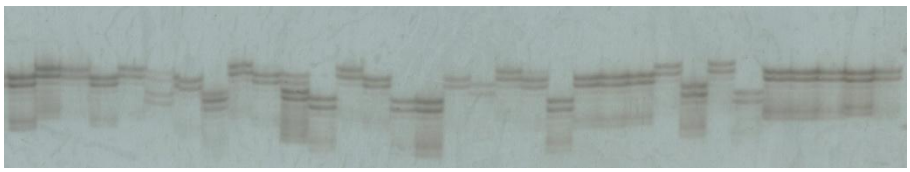

Figure 1- Pattern of the loci SG3D1 in 33 accessions of *S. guianensis.*

Polymorphism among the 150 accessions evaluated by the 20 microsatellites was estimated by calculating the number of alleles for each locus, the observed and expected heterozygosities and the Roger's genetic distance modified by Wright (1978) among all pairs of accessions. The number of alleles ranged from 2 to 11 (Table 2), with an average of 4.7 alleles per locus, totalizing 94 alleles. Vander Stappen et al. (1999b) observed a mean of 3.7 alleles per locus studying 65 genotypes of *S. guianensis* and some related species. Karia (2008) observed a mean of 6.43 alleles per locus using seven microsatellites and this higher value could be associated to the higher number of accessions studied (437). The observed and expected heterozygosities (H_O,H_E) ranged respectibely from 0 to 0.58 (mean of 0.18) and from 0.04 to 0.83 (mean of 0.55). The data revealed a deficit of heterozygous, which is in agreement with the outcrossing rate of 26% estimated for this species using microsatellites (Santos et al. unpubl. data). It should be emphasized that, in the case of bulked samples, a heterozygous pattern can occur by the presence of heterozygous plants in the DNA pool or by the presence of within accession variation (Zhang et al. 2008). Similar H_E values (0.57) were observed by Karia (2008), indicating that the set of plants evaluated here was representative of the genetic diversity present in this germplasm collection.

The model-based analysis was perfomed by STRUCTURE in order to investigate possible population structure in the 150 accessions. The number of clusters was set to nine according to the statistics of Evanno et al. (2005). In the ancestry plot (Fig. 2B), each accession is represented by a horizontal bar, and the length of each segment in the bar is proportional to the accession's

estimated ancestry fraction from each of the nine groups. The majority of the model-based groups were in agreement with the *S. guianensis* taxonomical classification (Ferreira and Costa 1979, Ferreira et al. 1985) and some of them could be related to the geographical origin of the accessions.

Table 2 – Results of the 20 microsatellites in 150 accessions of *S. guianensis*. Shown are the loci names, the number of alleles per locus (A), the observed (H_O) and expected (H_E) heterozygostities.

Loci	A	H_O	H_E
SG03A7	6	0.16	0.66
SG03B5	2	0.00	0.04
SG03D4	3	0.03	0.59
SG03E1	2	0.00	0.43
SG03D1	4	0.11	0.59
SG03G2	7	0.11	0.78
SG03G4	5	0.06	0.68
SG01B9	5	0.54	0.58
SG03G8	11	0.10	0.78
SG01C2	2	0.05	0.36
SG01B12	2	0.15	0.44
SG01D7	4	0.58	0.51
SG01D3	2	0.18	0.18
SG03A9	2	0.00	0.45
SG03E10	7	0.20	0.83
SG03B10	7	0.35	0.79
SG03E2	10	0.35	0.68
SG03E7	4	0.03	0.73
SG01A7	5	0.49	0.56
SG03E9	4	0.11	0.40

The *Stylosanthes* taxonomy is notoriously difficult mainly due to the absence of stable morphological markers (Vander Stappen et al., 1999a). *S. guianensis* is the most controversial group within this genus, mostly due to its great morphological variation and diverse number of species and varieties that have been proposed by several authors depending on what characters are considered to be suitable for species identification (Ferreira and Costa 1979, 't Manetje 1984, Ferreira et al. 1985). Here we consider the classification

proposed by Ferreira and Costa (1979) and Ferreira et al. (1985) with the nomenclatural modification made by (Costa 2006) in which this species is subdivided into the following four botanical varieties, *S. guianensis* var. *microcephala*, *S. guianensis* var. *guianensis*, *S. guianensis* var. *pauciflora* and *S. guianensis* var. *canescens*. This classification has been supported by studies using RAPDs (Kazan et al. 1993, Vieira et al. 1997), rDNA (Costa 2006) and cytogenetics (Vieira et al. 1993).

The distribution of the different varieties in the nine groups showed that the varieties *microcephala* and *canescens* were differentiated and formed individual groups, with few exceptions. On the other hand, several of the accessions belonging to the varieties *guianensis* and *pauciflora* formed distinct groups and, in addition, other groups were formed by a mixture of individuals from these two varieties. The mixed groups comprised small numbers of individuals and many of them were not classified into varieties. The *pauciflora* was recognized as a variety in 1985 (Ferreira et al. 1985) while the other three varieties were recognized earlier in 1979 (Ferreira and Costa 1979). This fact can have misled the classification, so that some of the accessions belonging to the variety *pauciflora* could have been classified as *guianensis* previously to the description of *pauciflora*. Besides, the proximity between these two varieties was previously reported by a cytogenetic study which showed that *pauciflora* and *guianensis* have similar karyotypes, differed only by the larger size of chromosome 10 in *pauciflora.* On the other hand, c*anescens* and *microcephala* differ from the other two varieties on the morphology of chromosome 10, being

microcephala karyotype smaller and more assymetrical than the ones from the other varieties (Vieira et al. 1993).

The number of accessions belonging to each variety assigned to one of the nine groups with more than 80% of membership probability is shown in Table 3. Out of the 40 accessions belonging to the *guianensis* variety, 11 were assigned to group C, five to group H and 14 to group I with a membership greater than 80%. These groups are formed mostly by accessions belonging to this variety, showing a great genetic diversity. The remaining 10 accessions were distributed together with *pauciflora* accessions. Three of the accessions were assigned to group B and two being assigned to group G, while five were not assigned to any group with more than 80% probability. Of the 28 *pauciflora* accessions, 13 were assigned to group A, which is formed mostly by this variety, seven were assigned together with *guianensis* in groups B and G, two were in distinct groups (E and I) and six were not assigned to any group with more than 80% of probability.

Table 3 - Inferred ancestry of the 150 accessions in relation of the groups obtained with Structure (A-I). Show are the proportion of individuals belonging to each botanical variety assigned to each of the nine groups and the number of individuals assigned to each group with more than 80% of membership (in bold).

Botanical Variety	A	B	C	D	E	F	G	H	I	p<0.80
S. guianensis var. *guianensis*	0,02	**3**/0.07	**11**/0.31	0,01	0.00	0,02	**2**/0.08	**5**/0.12	**14**/0.36	5
S. guianensis var. *microcephala*	**1**/0.07	0.00	0.00	**6**/0.40	**6**/0.40	0.00	0.00	0.00	**2**/0.13	0
S. guianensis var. *pauciflora*	**13**/0.59	**5**/0.21	0.00	0,01	**1**/0.05	0.00	**2**/0.07	0,01	**1**/0.06	6
S. guianensis var. *canescens*	0.01	0.01	0.02	0.00	0.01	**14**/0.79	0.00	0.00	**2**/0.13	4
Without Variety Identification	**3**/0.11	**1**/0.09	**1**/0.07	**1**/0.05	**3**/0.09	**6**/0.14	**4**/0.11	**1**/0.04	**13**/0.30	14

In previous studies most of the *microcephala* accessions did not cluster together (Faleiro et al. 2003, Karia 2008). In the present work, they were clustered in two diverse groups of accessions, one from states of Goiás and

Tocantins and another from the state of Minas Gerais. Of the 14 accessions of *microcephala*, six accessions from the state of Minas Gerais were assigned to group D and five accessions (two from the state of Goias and three from the state of Tocantins) were assigned to group E. In both groups, the majority of the accessions were *microcephala*. A similar geographical pattern was observed for the *microcephala* variety by Karia (2008) in which the soil of Goiás and Tocantins was considered less fertile than Minas Gerais. This difference in soil fertility could be the main cause of the observed genetic differentiation. The data reinforces the idea that *microcephala* occurrence in the State of Minas Gerais is associated with more fertile soils as proposed by Costa (2006), but further studies are necessary to address this question. The *canescens* accessions clustered together in a distinct group (F) composed exclusively by this variety. Of the 20 accessions of *canescens* studied, 14 were assigned to group F with more than 80% of probability.

Individuals that were not assigned to any group with more than 80% of membership probability were considered a recombination of different groups. Several of the studied accessions were not classified into botanical varieties and their distribution in clusters is shown in the Table 3. It can be observed that they were distributed in all groups. A proposal for the classification of the accessions based on clustering observed here could not be done with certainty, since some mixture of accession belonging to different varieties was observed.

The estimated Roger's genetic distances between accessions varied from 0 (among several accessions) to 0.94 (between accessions 19 and 140), with an average of 0.66. The accession 19 belongs to the variety *pauciflora* and was

collected in Venezuela, while the accession 140 belongs to the variety *guianensis*, and was collected in Brazil, in the State of Minas Gerais. The accession 140 was one of the most genetic distant accessions, with a mean genetic distance of 0.75, which is greater than the overall mean genetic distance (0.66). Faleiro et al. (2003) studied 35 accessions of *S. guianensis* using RAPD and observed genetic distances varying from 0.04 to 0.54. Kazan et al. (1993) studied 31 accessions of *S. guianensis* and observed a mean genetic distance of 0.26. Chang-Shun et al. (2004) studied 42 accessions of *S. guianensis* using AFLP markers and observed a genetic similarity ranging from 31 to 95%. The genetic distances observed here were higher than the ones previously reported for this species. Microsatellites are codominant multiallelic markers with a higher degree of polymorphism, thus more efficient in revealing diversity when compared to dominant markers, as AFLPs (Laborda et al. 2005). Even though, several accessions could not be distinguished with the 20 SSR markers used in this work. Karia (2008) observed 58 groups of accessions that were not differentiated, including one group of 26 putative replicates, possible due to the low number of markers used (7 SSRs). In contrast, the identical accessions observed here showed some genetic distance in the study reported by Karia (2008), thus the hypothesis of replicates was discarded.

The genetic differentiation among the *S. guianenis* groups clustered by the Bayesian approach was estimated based on Nei's G_{ST} as 0.46 and indicated that 46% of the differentiation was due to the variation among groups and the remaining 64% was a function of the genetic differentiation within groups. The observed G_{ST} was higher than the observed with allozymes in plants with a

mixed mating system (Hamrick and Godt 1996), probably because in *S. guianensis* there is a high predominance of autogamy (26% of outcrossing). High variation values (30%) had been observed between *S. humilis* groups of Mexican and South American accessions using AFLP (Vander Stappen et al. 2000). The variation was even higher in AFLP studies of *S. humilis* (59%) and *S. viscosa* (66%) (Sawkins et al. 2001). In general, G_{ST} values in autogamous or predominantly autogamous species are higher than in allogamous (Hamrick and Godt 1996, Maki et al. 2003). Similar G_{ST} values were observed between Andean and Mesoamerican groups of common bean (Zhang et al. 2008). As for wild barley, which is considered autogamous, the reported G_{ST} among populations was approximately 69% (Ozkan et al. 2005). The G_{ST} values among the groups clustered in the Bayesian analysis were higher than the ones observed for the mating system population.The accessions selected for this germplasm analysis are representative of the genetic diversity of the evaluated *S. guianensis* germplasm collection, and they were not related, whereas themating system was analysed in a population derived from 20 initial plants.

In the dendrogram constructed using DARwin and the UPGMA method (Fig.2A), the clusters, with few exceptions, were consistent with groups in the Bayesian approach of the STRUCTURE software. These data reinforces the classification proposed by Ferreira and Costa (1979) and Ferreira et al. (1985). Even though this classification was based on morphological characters, such as growth habit, it showed a strong genetic basis (Kazan et al. 1993, Vieira et al. 1997, Faleiro et al. 2003, Costa 2006, Karia 2008).

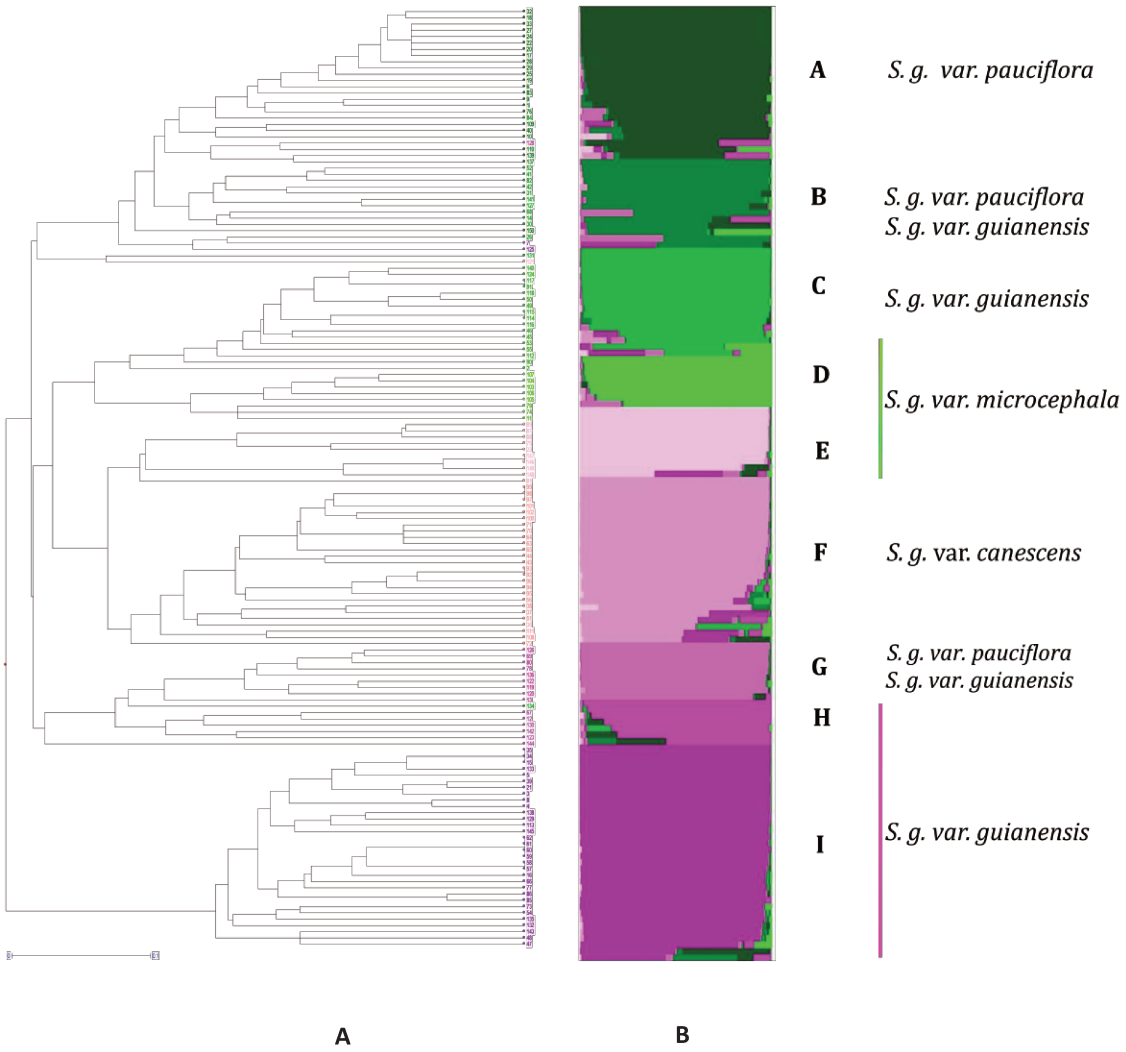

Figure 2 – A – Dendrogram obtained from the genetic distance of Roger's modified by Wright of 150 accession of *S. guianensis*, using the UPGMA method implemented in the software DARwin. B – Barplot obtained from the model-based analysis of ancestry of 150 accessions of *S. guianensis*, implemented in the software Structure.

The results showed here support the previous taxonomical classification from Ferreira and Costa (1979) and Ferreira et al. (1985) and give valuable information for the breeding program of *S. guianensis*. The knowledge about the amount and distribution of the genetic diversity present in the germplasm collection is a key for efficient germplasm preservation and utilization by breeders.

References

Alcaraz M, Hormaza J. 2007. Molecular characterization and genetic diversity in an avocado collection of cultivars and local Spanish genotypes using SSRs. Hereditas 144: 244-253.

Barkley NA, Roose ML, Krueger RR, Federici CT. 2006. Assessing genetic diversity and population structure in a citrus germplasm collection utilizing simple sequence repeat markers (SSRs). Theoretical and applied genetics. 112: 1519-31.

Barrett S. 2003. Mating strategies in flowering plants: the outcrossing-selfing paradigm and beyond. Philosophical transactions of the Royal Society of London. Series B, Biological sciences 358: 991-1004.

Bellon G, Faleiro FG, Ferreira CF, Karia CT, Fonseca KG, Santos JR, Teixeira MA, Junqueira KP. 2007. Validação e otimização de protocolo simplificado de extração de DNA a partir de tecido foliar. Congresso Brasileiro de Melhoramento de Plantas. São Lourenço: Sociedade Brasileira de Melhoramento de Plantas, 1-5.

Blair M, Diaz J, Hidalgo R, Diaz L, Duque M. 2007. Microsatellite characterization of Andean races of common bean (*Phaseolus vulgaris* L.). Theoretical and Applied Genetics 116: 29-43.

Chang-Shun J, Hu-Sen J, Xin-Rong M, Dong-Mei Z, Yi-Zheng Z. 2004. AFLP analysis of genetic variability among *Stylosanthes guianensis* acessions resitant and susceptible to stylo anthracnose. Acta Botanica Sinica 46: 480-488.

Cordeiro G, Pan Y, Henry RJ. 2003. Sugarcane microsatellites for the assessment of genetic diversity in sugarcane germplasm. Plant Science 165: 181-189.

Costa N. 2006. Revisão do género *Stylosanthes*. phD thesis. Universidade Técnica de Lisboa : 470p.

Creste S, Tulmann Neto A, Figueira A. 2001. Detection of single sequence repeat polymorphisms in denaturing polyacrylamide sequencing gels by silver staining. Plant Molecular Biology Reporter 19: 299-306.

Doyle J, Doyle J. 1990. Isolation of plant DNA from fresh tissue. Focus 12: 13-15.

Edye L, Cameron D. 1984. Prospects for *Stylosanthes* improvement and utilization. In: Stace, H, Edye, L, The biology and agronomy of Stylosanthes. Sydney: Academic Press, 571-588.

Evanno G, Regnaut S, Goudet J. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular ecology 14: 2611-20.

Faleiro FG, Karia CT, Andrade RP, Barros AM, Silva DO. 2003. Diversidade genética de uma coleção de trabalho de *Stylosanthes guianensis* com base em marcadores RAPD. Reunião Anual da Sociedade Brasileira de Zootecnia. Santa Maria: SBZ, 1.

Ferreira M, Costa N. 1979. O genero *Stylosanthes* Sw. no Brasil. Belo Horizonte: EPAMIG.

Ferreira M, Costa N, Schultze-Kraft K. 1985. Pauciflora, uma nova variedade de *Stylosanthes guianensis* (Aubl.) Sw. Anais do Congresso Nacional de Botanica. 235-241.

Goudet J. 2001. FSTAT (Version 1.2): a computer program to calculate F-statistics. Available at: http://www2.unil.ch/popgen/softwares/fstat.htm.

Hamrick J, Godt M. 1996. Effects of life history traits on genetic diversity in plant species. Philosophical Transactions of the Royal Society B 351: 1291-1298.

Hamrick J, Linhart Y, Mitton J. 1979. Relationship between life history parameters and allozyme variation in plants. Annual review of ecology and systematics 10: 173-200.

Karia CT. 2008. Caracterização genética e morfoagronomica de germoplasma de *Stylosanthes guianensis* (Aubl.) Sw. phD thesis. Universidade Federal de Goiás: 140p.

Kazan K, Manners JM, Cameron DF. 1993. Genetic relationships and variation in the *Stylosanthes guianensis* species complex assessed by random amplified polymorphic DNA. Genome 36: 43-49.

Kelemu S, Changshun J, Guixi H, Segura G. 2005. Genetic transformation of the tropical forage legume *Stylosanthes guianensis* with rice-chitinase gene confers resistance to *Rhizoctonia* foliar blight disease. African Journal of Biotechnology 4: 1025-1033.

Krishna GK, Zhang J, Burow M, Pittman RN, Delikostadinov SG, Lu Y NP. 2004. Genetic diversity analysis in valencia peanut (*Arachis hypogaea* L.) using microsatellite markers . Cellular and Molecular Biology Letters 9: 685 - 697.

Laborda PR, Oliveira KM, Garcia AA, Paterniani ME, Souza AP. 2005. Tropical maize germplasm: what can we say about its genetic diversity in the light of molecular markers? Theoretical and Applied Genetics 111: 1288-1299.

Lewis PO, Zaykin D. 2000. Genetic Data Analysis: Computer program for the analysis of allelic data. Available at: http://alleyn.eeb.uconn.edu/gda/ 2000.

Maki M, Yamashiro T, Matsumura S. 2003. High levels of genetic diversity in island populations of the island endemic *Suzukia luchuensis* (Labiatae). Heredity 91: 300-6.

Maass BL, Sawkins MC. 2004. History, relationships and diversity among *Stylosanthes* species of commercial significance. In: Chakraborty, S, High-yielding anthracnose-resistant *Stylosanthes* for agricultural systems. Sydney: CSIRO, 9-26.

Miller MP. 1997. Tools for population genetic analysis (TFPGA) : A Windows program for the analysis of allozyme and molecular population genetic data. Available at: http://www.marksgeneticsoftware.net/tfpga.htm

Ozkan H, Kafkas S, Ozer M, Brandolini A. 2005. Genetic relationships among South-East Turkey wild barley populations and sampling strategies of *Hordeum spontaneum*. Theoretical and applied genetics (TAG) 112: 12-20.

Pathak P, Ramesh C, Bhatt R. 2004. *Stylosanthes* in the reclamation and development of degraded soils in India. In: S, C, High-yielding anthracnose-resistant Stylosanthes for agricultural systems. Canberra: Australian Centre for International Agricultural Research, 85-96.

Perrier X, Jacquemoud-Collet J. 2006. DARwin software. Available at: http://darwin.cirad.fr/darwin.

Pritchard JK, Stephens M, Donnelly P. 2000. Inference of Population Structure Using Multilocus Genotype Data. Genetics 155: 945-959.

Santos MO, Karia CT, Resende RM, Chiari L, Jungmann L, Zucchi MI, Souza AP. 2009. Isolation and characterization of microsatellite loci in the tropical forage legume *Stylosanthes guianensis* (Aubl.) Sw. Conservation Genetics Resources. Online first, DOI: 10.1007/s12686-009-9010-2.

Sawkins MC, Maass BL, Pengelly C, Newburry HJ, Ford-Lloyd BV, Maxted N, Smith R. 2001. Geographical patterns of genetic variation in two species of *Stylosanthes* Sw. using amplified fragment lenght polymorphism. Molecular Ecology: 1947-1958.

Stace H. 1982. Breeding systems in *Stylosanthes*. Observations of outcrossing in *S. scabra* at an alcohol dehydrogenase locus. Australian Journal of Agricultural Research 33: 87-96.

Stace H, Cameron D. 1984. Cytogenetics and the evolution of Stylosanthes. In: Stace, H, Edye, L, The biology and agronomy of *Stylosanthes*. Sydney: Academic Press, 49-72.

t' Manetje L. 1984. Considerations on the taxonomy of the genus *Stylosanthes*. In: Stace, H, Edye, L, The biology and agronomy of Stylosanthes. Sydney: Academic Press, 1-22.

Vander Stappen J, Weltjens I, Lopez S, Volckaert G. 2000. Genetic diversity in Mexican *Stylosanthes humilis* as revealed by AFLP, compared to the variabiliy of *S. humilis* accessions of South American origin. Euphytica 113: 145-154.

Vander Stappen J, Weltjens I, Van Campenhout S, Volckaert G. 1999a. Genetic relationships among *Stylosanthes* species as revealed by sequence-tagged site markers. Theoretical and Applied Genetics 98: 1054-1062.

Vander Stappen J, Weltjens I, Volckaert G. 1999b. Microsatellite markers in *Stylosanthes guianensis*. Molecular Ecology 8: 514-517.

Vieira MLC, Aguiar-perecin MLR, Martins OS. 1993. A cytotaxonomic study in twelve brazilian taxa of Stylosanthes Sw., Leguminosae. Cytologia 58: 305-311.

Vieira MLC, Fungaro MH, Jubier MF, Lejeune B. 1997. Determination of taxonomic relationships among Brazilian taxa of *Stylosanthes* Sw., Leguminosae, using RAPD markers. Pesquisa Agropecuária Brasileira 32: 305-310.

Westman AL, Kresovich S. 1997. Use of molecular marker techniques for description of plant genetic variation. . In: Callow, JA, Ford-Lloyd, BV, Newbury, JH, Plant Genetic resources. Conservation and Use. Wallingford: CAB International, 9-48.

Williams RJ, Reid R, Schultze-Kraft R, Costa NM, Thomas BD. 1984. Natural distribution of *Stylosanthes*. In: Stace, H, Edye, L, The biology and agronomy of *Stylosanthes*. Sydney: Academic Press, 73-101.

Wright S. 1978. Evolution and the genetics of populations, vol.4. Variability within and among natural populations. Chicago: University of Chicago Press. 590p.

Zhang X, Blair MW, Wang S. 2008. Genetic diversity of Chinese common bean (*Phaseolus vulgaris* L.) landraces assessed with simple sequence repeat markers. Theoretical and Applied Genetics 117: 629-40.

Zong X, Redden RJ, Liu Q, Wang S, Guan J, Liu J, Xu Y, Liu X, Gu J, Yan L, Ades P, Ford R. 2009. Analysis of a diverse global *Pisum* sp. collection and comparison to a Chinese local *P. sativum* collection with microsatellite markers. Theoretical and applied genetics 118: 193-204.

10 Conclusões

10.1 Desenvolvimento dos Marcadores Microssatélites

- ✓ A metodologia empregada na construção da biblioteca enriquecida foi eficiente nas três espécies estudadas e possibilitou o desenvolvimento de marcadores polimórficos que podem ser utilizados na avaliação da diversidade genética de coleções, no estudo de estrutura de populações naturais, na distinção de variedades comerciais, entre outras aplicações.

- ✓ *S. macrocephala* foi a espécie que apresentou o menor valor de heterozigosidade observada (0,09) e esperada (0,40), sendo a espécie estudada que apresenta a distribuição geográfica mais restrita. *S. guianensis*, a espécie com a mais ampla distribuição geográfica foi a que apresentou o maior valor de heterozigosidade esperada (0,55).

- ✓ O resultado dos testes de transferibilidade dos marcadores entre as espécies de *Stylosanthes* concordou com os dados prévios de filogenia, sendo que os marcadores de *S. guianensis*, a espécie mais distante filogeneticamente, foram os que mostraram a menor taxa de transferibilidade para as outras espécies.

10.2 Estudo da Taxa de Cruzamento

- ✓ A estimativa da taxa de cruzamento para *S. capitata* e *S. guianensis* indicou a presença de um sistema misto de cruzamento com predominância de autogamia.

- ✓ O conhecimento da taxa de cruzamento é de grande importância para o melhoramento, para a multiplicação de sementes e para a manutenção de bancos de germoplasma. A taxa de cruzamento observada para *S. capitata* e *S. guianensis* vão possibilitar a escolha de métodos de melhoramento adequados à espécies de sistema misto. Essa taxa de cruzamento também deve ser considerada durante a multiplicação de sementes para previnir a mistura de diferentes acessos e no planejamento de coletas, visando amostrar melhor a variabilidade genética.

10.3 Estudo da Diversidade Genética em Bancos de Germoplasma

- ✓ Os microssatélites desenvolvidos se mostraram úteis na avaliação da diversidade genética de bancos de germoplasma de *S. guianensis* e *S. macrocephala*.

- ✓ Em *S. guianensis* foram avaliados 150 acessos escolhidos para representar o banco de germoplasma da Embrapa Cerrados, com base

nos agrupamentos observados em um trabalho anterior que avaliou 437 acessos dessa mesma coleção. Foi possível relacionar os grupos formados através da análise dos microssatélites com as diferentes variedades botânicas propostas com base em caracteres morfológicos. Uma das variedades botânicas mostrou dois pools genéticos distintos relacionados à região de coleta dos acessos, que apresenta tipos de solos distintos. No geral, os resultados estão de acordo com dados taxonômicos, citogenéticos e moleculares publicados anteriormente e podem ser utilizados na escolha de parentais para cruzamento e na seleção de acessos para avaliações agronômicas.

- ✓ Em *S. macrocephala*, foram avaliados 134 acessos pertencentes ao banco de germoplasma da Embrapa Cerrados. A análise dos marcadores levou a formação de cinco grupos genéticos distintos, mostrando uma ampla diversidade genética na coleção. Além disso, os dados indicam um possível centro de origem para as populações dessa espécie no estado da Bahia.

- ✓ Em ambas as espécies, a distância genética observada foi maior que a observada a partir de outros marcadores moleculares nessas espécies e em outras espécies de *Stylosanthes*, mostrando a utilidade dos microssatélites em estudos de diversidade genética.

11 Referências Bibliográficas

Abbott RJ, Gomes MF. 1989. Population genetic structure and outcrossing rate of *Arabidopsis thaliana* (L.) Heynh. Heredity 62: 411-418.

Abdel-Ghani AH, Parzies HK, Omary A, Geiger HH. 2004. Estimating the outcrossing rate of barley landraces and wild barley populations collected from ecologically different regions of Jordan. Theoretical and applied genetics 109: 588-595.

Alcaraz M, Hormaza J. 2007. Molecular characterization and genetic diversity in an avocado collection of cultivars and local Spanish genotypes using SSRs. Hereditas 144: 244-253.

Álvarez I, Wendel JF. 2003. Ribosomal ITS sequences and plant phylogenetic inference. Molecular Phylogenetics and Evolution 29: 417-434.

Andrade RP, Karia CT. 2000. Uso de *Stylosanthes* em pastagens no Brasil. Anais do Simpósio de Forragicultura e Pastagens: Temas em evidência. Lavras: UFLA, 471-475.

Andrade RP, Karia CT, Ramos AK. 2004. *Stylosanthes* as a forage legume as its centre of diversity. In: Chakraborty, S, High-yielding anthracnose-resistant *Stylosanthes* for agricultural systems. Canberra: CSIRO, 39-50.

Asfaw A, Blair MW, Almekinders C. 2009. Genetic diversity and population structure of common bean (*Phaseolus vulgaris* L.) landraces from the East African highlands. Theoretical and applied genetics. Online first, DOI: 10.1007/s00122-009-1154-7.

Barcellos AO, Andrade RP, Zoby JL, Vilelaa L. 2001. Bancos de proteínas de *Stylosanthes guianensis* cv Mineirão: Maneira simples e de baixo custo para fornecer proteínas ao gado na seca. Circular Técnica Embrapa 14: 1-5.

Barker GL, Edwards KJ. 2009. A genome-wide analysis of single nucleotide polymorphism diversity in the world's major cereal crops. Plant Biotechnology Journal: 318-325.

Barros AM, Faleiro FG, Karia CT, Shiratsuchi LS, Andrade RP, Lopes GK. 2005. Variabilidade genética e ecológica de *Stylosanthes macrocephala* determinadas por RAPD e SIG. Pesquisa Agropecuária Brasileira 40: 899-909.

Batley J, Barker G, Sullivan HO, Edwards KJ, Edwards D, Victoria A, Centre PB. 2003. Mining for Single Nucleotide Polymorphisms and Insertions/Deletions in Maize Expressed Sequence Tag Data. Plant Physiology 132: 84-91.

Beland JD, Krakowski J, Ritland CE, Ritland K, El-Kassaby YA. 2005. Genetic structure and mating system of northern *Arbutus menziesii* (Ericaceae) populations. Canadian Journal of Botany 83: 1581–1589.

Blair MW, Díaz LM, Buendía HF, Duque MC. 2009. Genetic diversity, seed size associations and population structure of a core collection of common beans (*Phaseolus vulgaris* L.). Theoretical and applied genetics. Online first, DOI: 10.1007/s00122-009-1064-8.

Borém AB, Miranda GV. 2005. Melhoramento de plantas. 4 ed. Viçosa: Editora UFV. 525p.

Botstein D, White RL, Skolnick M, Davis RW. 1980. Construction of a genetic linkage map in man using restriction fragment lenght polymorphisms. American Journal of Human Genetics 32: 314-331.

Bray R, Hutton E. 1976. Plant breeding and genetics. Tropical Pasture Research 51: 388-353.

Brown AH. 1990. Genetic characterization of plant mating system. In: Hamrick, JL, Godt, MJ, Brown, AH, Clegg, MT, Plant population genetics, breeding and genetic resources. Sunderland: Sinauer, 155-162.

Brown AH, Allard RW. 1970. Estimation of the mating system in open-pollinated maize populations using isozime polymorphisms. Genetics 66: 133-145.

Brown AH, Zohary D, Nevo E. 1978. Outcrossing rates and heterozygosity in natural populations of *Hordeum spontaneum* Koch in Israel. Heredity 41: 49-62.

Bundock PC, Henry RJ. 2004. Single nucleotide polymorphism, haplotype diversity and recombination in the Isa gene of barley. Theoretical and applied genetics 109: 543-51.

Cameron D, Chakraborty S. 2004. Forage potencial of *Stylosanthes* in different production systems. In: Chakraborty, S, High-yielding anthracnose-resistant *Stylosanthes* for agricultural systems. Canberra: CSIRO, 27-38.

Chandra A. 2006. A Partial Genetic Map of *Stylosanthes* Developed Using Inter-Specific F2 Cross. Cytologia 71: 353-358.

Chang-Shun J, Hu-Sen J, Xin-Rong M, Dong-Mei Z, Yi-Zheng Z. 2004. AFLP analysis of genetic variability among *Stylosanthes guianensis* acessions resitant and susceptible to stylo anthracnose. Acta Botanica Sinica 46: 480-488.

Charlesworth D, Wright SI. 2001. Breeding systems and genome evolution. Current Opinion in Genetics and Development 11: 685-690.

Cho RJ, Mindrinos M, Richards DR, Sapolsky RJ, Anderson M, Drenkard E, Dewdney J, Reuber TL, Stammers M, Federspiel N, Lemieux B, Dean C, Lipshutz RJ, Ausubel FM, Davis RW. 1999. Genome-wide mapping with biallelic markers in *Arabidopsis thaliana*. Nature Genetics 23: 203-207.

Clegg M. 1980. Measuring plant mating systems. Bioscience 30: 814–818.

Costa N. 2006. Revisão do género Stylosanthes. PhD thesis. Universidade Técnica de Lisboa : 470p.

Costa NM, Ferreira MB. 1984. Some Brazilian species of *Stylosanthes*. In: Stace, HM, Edye, L, The biology and agronomy of *Stylosanthes*. Sydney: Academic Press, 23-48.

Djè Y, Heuertz M, Ater M, Lefebvre C, Vekemans X. 2004. In situ estimation of outcrossing rate in sorghum landraces using microsatellite markers. Euphytica 138: 205–212.

Djè Y, Heuertz M, Lefèbvre C, Vekemans X. 2000. Assessment of genetic diversity within and among germplasm accessions in cultivated sorghum using microsatellite markers. Theoretical and Applied Genetics 100: 918-925.

EMBRAPA Gado de Corte. 2000. Estilosanthes Campo Grande. http://www.cnpgc.embrapa.br/publicacoes/divulga/GCD38.html. Acessado em 11/09/09.

Ferreira M, Costa N. 1979. O genero *Stylosanthes* Sw. no Brasil. Belo Horizonte: EPAMIG.

Ferreira ME, Grattapaglia D. 1998. Introdução ao uso de marcadores moleculares em análise genética. Brasília: Embrapa. 220p.

Ferreira M, Costa N, Schultze-Kraft K. 1985. Pauciflora, uma nova variedade de *Stylosanthes guianensis* (Aubl.) Sw. Anais do Congresso Nacional de Botanica. 235-241.

Franco JB, Rosa R. 2003. Metodologia de coleta de dados radiométricos em pastagens do gênero *Brachiaria* com diferentes estágios de degradação. Anais do XI Simpósio Brasileiro de Sensoriamento Remoto. Belo Horizonte, 2483-2491.

Gaiotto FA, Bramucci M, Grattapaglia D. 1997. Estimation of outcrossing rate in a breeding population of *Eucalyptus urophylla* with dominant RAPD and AFLP markers. Theoretical and Applied Genetics 95: 842-849.

Ganal MW, Altmann T, Röder MS. 2009. SNP identification in crop plants. Current Opinion in Plant Biology 12: 211-7.

Gardiner JM, Coe EH, Melia-Hancock SD, Hoisington DA, Chao S. 1993. Development of a Core RFLP Map in Maize Using an Immortalized F(2) Population. Genetics 134: 917-930.

Gillies AC, Abbott RJ. 1996. Phylogenetic relationships in the genus *Stylosanthes* (Leguminosae) based upon chloroplast DNA variation. Plant Systematics and Evolution 200: 1615-6110.

Graner A, Jahoor A, Schondelmaier J, Siedler H, Pillen K, Fischbeck G, Herrmann G, Wenzel G, Herrmann RG. 1991. Construction of a RFLP map in barley. Theoretical and Applied Genetics 83: 1432-2242.

Grof B, Schultze-Kraft R, Miller F. 1979. *Stylosanthes capitata* Vog., some agronomic attributes, and resistance to anthracnose *Colletotrichum gloeosporioides* Penz. Tropical Grasslands 13: 28-37.

Guimarães CT, Moreira MA. 1999. Genética molecular aplicada ao melhoramento de plantas. In: Borém A, Melhoramento de espécies cultivadas. Viçosa: UFV, 715-740.

Gupta PK, Roy JK, Prasad M. 2001. Single nucleotide polymorphisms: A new paradigm for molecular marker technology and DNA polymorphism detection with emphasis on their use in plants. Current Science 80: 526-535.

Hamada H, Petrino MG, Kakanaga T. 1982. A novel repeated element with Z-DNA forming potential is widely found in evolutionarily diverse eukaryotic genomes. Proceedings of the National Academy of Science USA 79: 6465-6469.

Hamrick J, Godt M. 1996. Effects of life history traits on genetic diversity in plant species. Philosophical Translactions of the Royal Society B 351: 1291-1298.

Hao CY, Zhang XY, Wang LF, Dong YS, Shang XW, Jia JZ. 2006. Genetic Diversity and Core Collection Evaluations in Common Wheat Germplasm from the Northwestern Spring Wheat Region in China. Molecular Breeding 17: 69-77.

Horres R, Zizka G, Kahl G, Weising K. 2008. Molecular Phylogenetics of Bromeliaceae: Evidence from trnL(UAA) Intron Sequences of the Chloroplast Genome. Plant Biology 2: 306-315.

Huang Q, Börner a, Röder S, Ganal W. 2002. Assessing genetic diversity of wheat (*Triticum aestivum* L.) germplasm using microsatellite markers. Theoretical and applied genetics 105: 699-707.

IBGE. 2006. Censo Agropecuário. Disponível em /http://www.ibge.gov.br/home/estatistica/economia/agropecuaria/censoagro/2006/defaulttab_censoagro.shtm. Acessado em 11/09/2009.

Kahrood HV, Korori SA, Pirseyedi M, Shirvany A, Danehkar A. 2008. Genetic variation of mangrove species *Avicennia marina* in Iran revealed by microsatellite markers. African Journal of Biotechnology 7: 3017-3021.

Karia CT. 2008. Caracterização genética e morfoagronomica de germoplasma de Stylosanthes guianensis (Aubl.) Sw. PhD Thesis. Universidade Federal de Goiás: 140p.

Karia CT, Andrade RP. 1996. Avaliação preliminar de espécies forrageiras no Centro de Pesquisa Agropecuária do Cerrado: perspectivas futuras. International Symposium on Tropical Savannas. Brasília, 471-475.

Kazan K, Manners JM, Cameron DF. 1993. Genetic relationships and variation in the *Stylosanthes guianensis* species complex assessed by random amplified polymorphic DNA. Genome 36: 43-49.

Kirkbride JH, Kirkbride MC. 1985. Typification of Stylosanthes (Leguminosae) and its sections. Taxon 36: 455-458.

Litt M, Luty JA. 1989. A hypervariable microsatellite revealed by in vitro amplification of dinucleotide repeat within the cardiac muscle actin gene. American Journal of Human Genetics 44: 398-401.

Liu CJ. 1997. Geographical distribution of genetic variation in *Stylosanthes scabra* revealed by RAPD analysis. Euphytica 98: 21-27.

Liu C, Musial J, Thomas B. 1999. Genetic relationship among *Stylosanthes* species revealed by RFLP and STS analyses. Theoretical and Applied Genetics 99: 1179-1186.

Macedo MC, Zimmer AH. 1993. Sistema pasto-lavoura e seus efeitos na produtividade agropecuária. Anais do II Simpósio sobre Ecossistema de Pastagens. Jaboticabal: FUNESP, 216-245.

Macedo MC, Kichel AN, Zimmer AH. 2000. Degradação e alternativas de recuperação e renovação de pastagens. Embrapa Gado de Corte/Comunicado Técnico 62: 1-4.

Mackill DJ, Zhang Z, Redoña ED, Colowit PM. 1996. Level of polymorphism and genetic mapping of AFLP markers in rice. Genome 39: 969-977.

Maass BL, Sawkins MC. 2004. History, relationships and diversity among *Stylosanthes* species of commercial significance. In: Chakraborty, S, High-yielding anthracnose-resistant *Stylosanthes* for agricultural systems. Sydney: CSIRO, 9-26.

Miles J. 1983. Natural outcrossing in *Stylosanthes capitata*. Tropical Grasslands 17: 114-117.

Miles J. 1985. Evaluation of potential genetic marker traits and estimation of outcrossing rate in *Stylosanthes guianensis*. Australian Journal of Agricultural Research 36: 259-265.

Mogg R, Batley J, Hanley S, Edwards D, O'Sullivan H, Edwards J. 2002. Characterization of the flanking regions of *Zea mays* microsatellites reveals a

large number of useful sequence polymorphisms. Theoretical and applied genetics 105: 532-543.

Mullis K, Faloona F. 1987. Specific synthesis of DNA in vitro via polymerase catalyzed chain reaction. Methods in Enzymology 155: 335.

Newbury JH, Ford-Lloyd BV. 1993. The use of RAPD to assess variation in plants. Plant Growth Regulation 12: 43-51.

O'Connell LM, Russell J, Ritland K. 2004. Fine-scale estimation of outcrossing in western redcedar with microsatellite assay of bulked DNA. Heredity 93: 443-9.

Palmer JD. 1992. Mitochondrial DNA in plant systematics: applications and limitations. In: Soltis, PS, Soltis, DE, Doyle, JJ, Molecular Systematics of Plants. New York: Chapman and Hall, 36-49.

Pathak P, Ramesh C, Bhatt R. 2004. *Stylosanthes* in the reclamation and development of degraded soils in India. In: S, C, High-yielding anthracnose-resistant *Stylosanthes* for agricultural systems. Canberra: Australian Centre for International Agricultural Research, 85-96.

Peleg Z, Fahima T, Abbo S, Krugman T, Saranga Y. 2008. Genetic structure of wild emmer wheat populations as reflected by transcribed versus anonymous SSR markers. Genome 51: 187-195.

Phaikaew C, Ramesh C, Kexian Y, Stur W. 2004. Utilisation of *Stylosanthes* as a forage crop in Asia. In: Chakraborty, S, High-yielding anthracnose-resistant *Stylosanthes* for agricultural systems. Canberra: CSIRO, 65-76.

Prasad M, Varshney RK, Roy JK, Balyan HS, Gupta PK. 2000. The use of microsatellites for detecting DNA polymorphism, genotype identification and genetic diversity in wheat. Theoretical and Applied Genetics 100: 584-592.

Rafalski A, Tingey S. 2008. SNPs and their use in maize. In: Henry, R, Plant Genotyping II. CAB International, 30-43.

Resende RM, Resende MD, Jank L, Valle CB, Cançado LJ, Chiari L. 2008. Melhoramento genético de leguminosas forrageiras. In: Resende, RM, Valle, CB, Jank, L, Melhoramento de forrageiras tropicais. Campo Grande: Embrapa Gado de Corte, 117-133.

Reusch TB. 2000. Pollination in the marine realm: microsatellites reveal high outcrossing rates and multiple paternity in eelgrass *Zostera marina*. Heredity 85: 459-64.

Santos-Filho LS. 1996. Seed production: perspectives from the Brazilian private sector. In: Miles, JW, Maass, BL, Valle, CB, *Brachiaria*: biology, agronomy and improvement. Cali: CIAT-Embrapa, 141-146.

Sawkins MC, Maass BL, Pengelly C, Newburry HJ, Ford-Lloyd BV, Maxted N, Smith R. 2001. Geographical patterns of genetic variation in two species of *Stylosanthes* Sw. using amplified fragment lenght polymorphism. Molecular Ecology 10: 1947-1958.

Shaw D, Kahler A, Allard R. 1981. A multilocus estimator of mating system parameters in plant populations. Proceedings of the National Academy of Sciences USA 78: 1298–1302.

Souza AP. 2001. Biologia molecular aplicada ao melhoramento. In: Nass, LL, Valois, AC, Melo, IS, Valadares-Inglis, MC, Recursos genéticos e melhoramento. Rondonópolis: Fundação MT, 939-965.

Souza L, Kageyama P, Sebbenn A. 2003. Sistema de reprodução em população natural de *Chorisia speciosa* A. St.-Hil.(Bombacaceae). Revista Brasileira de Botânica 26: 113–121.

Stace H. 1982. Breeding systems in *Stylosanthes*. Observations of outcrossing in *S. scabra* at an alcohol dehydrogenase locus. Australian Journal of Agricultural Research 33: 87-96.

Stace H, Cameron D. 1984. Cytogenetics and the evolution of *Stylosanthes*. In: Stace, H, Edye, L, The biology and agronomy of *Stylosanthes*. Sydney: Academic Press, 49-72.

Tautz D, Renz M. 1984. Simple sequences are ubiquitous repetitive components of eukaryotic genomes. Nucleic Acids Research 12: 4127-4138.

Temnykh S, Park WD, Ayres N, Cartinhour S, Hauck N, Lipovich L, Cho YG, Ishii T, McCouch SR. 2000. Mapping and genome organization of microsatellite sequences in rice (*Oryza sativa* L.). Theoretical and Applied Genetics 100: 1432-2242.

Thumma BR, Naidu BP, Chandra A, Cameron DF, Bahnisch LM, Liu C. 2001. Identification of causal relationships among traits related to drought resistence in *Stylosanthes scabra* using QTL analysis. Journal of Experimental Botany 52: 203-214.

t' Manetje L. 1984. Considerations on the taxonomy of the genus *Stylosanthes*. In: Stace, H, Edye, L, The biology and agronomy of *Stylosanthes*. Sydney: Academic Press, 1-22.

Tropical Forages. 2005. An interactive selection tool. Disponível em: http://www.tropicalforages.info/index.htm. Acessado em 12/09/2009.

Valois AC, Nass LL, Goes M. 2001. Conservação ex-situ de recursos genéticos vegetais. In: Nass, LL, Valois, AC, Melo, IS, Valadares-Inglis, MC, Recursos genéticos e melhoramento. Rondonópolis: Fundação MT, 123-147.

Vander Stappen J, De Laet J, Gama-López S, Van Campenhout S, Volckaert G. 2002. Phylogenetic analysis of *Stylosanthes* (Fabaceae) based on the internal transcribed spacer region (ITS) of nuclear ribosomal DNA. Plant Systematics and Evolution 234: 1615-6110.

Vander Stappen J, Weltjens I, Gama-López S, Volckaert G. 2000. Genetic diversity in Mexican S. humilis as revealed by AFLP, compared to the variability of S. humilis accessions of South American origin. Euphytica 113: 145-154.

Vander Stappen J, Van Campenhout S, Gama Lopez S, Volckaert AG. 1999a. Sequencing of the internal transcribed spacer region ITS1 as a molecular tool detecting variation in the *Stylosanthes guianensis* species complex. Theoretical and Applied Genetics 96: 869-877.

Vander Stappen J, Weltjens I, Van Campenhout S, Volckaert G. 1999b. Genetic relationships among *Stylosanthes* species as revealed by sequence-tagged site markers. Theoretical and Applied Genetics 98: 1054-1062.

Vander Stappen J, Weltjens I, Volckaert G. 1999c. Microsatellite markers in *Stylosanthes guianensis*. Molecular Ecology 8: 514-517.

Vaquero F, Vences FJ, García P, Ramírez L, Pérez de la Vega M. 1989. Mating system in rye: variability in relation to the population and plant density. Heredity 62: 17-26.

Vieira MLC, Aguiar-perecin MLR, Martins OS. 1993. A cytotaxonomic study in twelve brazilian taxa of Stylosanthes Sw., Leguminosae. Cytologia 58: 305-311.

Vieira ML, Fungaro MH, Jubier MF, Lejeune B. 1997. Determination of taxonomic relationships among Brazilian taxa of *Stylosanthes* Sw., Leguminosae, using RAPD markers. Pesquisa Agropecuária Brasileira 32: 305-310.

Virk PS, Ford-Lloyd BV, Jackson MT, Newbury JH. 1995. Use of RAPD for the study of diversity within plant germplasm collections. Heredity 74: 170-179.

Vuylsteke MR, Mank R, Antonise R, Bastiaans E, Senior ML, Stuber CW, Melchinger AE, Lübberstedt TX, Xia C, Stam P, Zabeau M, Kuiper M. 1999. Two high-density AFLP linkage maps of *Zea mays* L.: analysis of distribution of AFLP markers. Theoretical and Applied Genetics 99: 921-935.

Westman AL, Kresovich S. 1997. Use of molecular marker techniques for description of plant genetic variation. . In: Callow, JA, Ford-Lloyd, BV, Newbury, JH, Plant Genetic resources. Conservation and Use. Wallingford: CAB International, 9-48.

Williams JG, Kubelik AR, Livak KJ, Rafalski LA, Tingey SV. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research 18: 6531-6535.

Williams RJ, Reid R, Schultze-Kraft R, Costa NM, Thomas BD. 1984. Natural distribution of *Stylosanthes*. In: Stace, H, Edye, L, The biology and agronomy of *Stylosanthes*. Sydney: Academic Press, 73-101.

Zhu J, Gale MD, Quarrie S, Jackson MT, Bryan GJ. 1998. AFLP markers for the study of rice biodiversity. Theoretical and Applied Genetics 96: 1432-2242.

Zong X, Redden RJ, Liu Q, Wang S, Guan J, Liu J, Xu Y, Liu X, Gu J, Yan L, Ades P, Ford R. 2009. Analysis of a diverse global *Pisum* sp. collection and comparison to a Chinese local *P. sativum* collection with microsatellite markers. Theoretical and applied genetics 118: 193-204.

Cidade Universitária "Zeferino Vaz",
09 de dezembro de 2009.

CIBio: 01/2009

Projeto CIBio: 14/2003

Identificação:
03/2005 – **Doutorado**: Melissa Oliveira Santos, Bióloga, CPG-GBM UNICAMP
Projeto: Desenvolvimento de marcadores moleculares do tipo microssatélites para forrageiras leguminosas de importância econômica no Brasil visando aplicações no melhoramento.

Parecer:
Projeto aprovado pela CIBio/CBMEG em 13/02/2003 sob número 14/2003 (em andamento)
Coordenador: Profa. Dra. Anete Pereira de Souza

Profa. Dra. Edi Lúcia Sartorato
Presidente da CIBio/CBMEG - UNICAMP

UNIVERSIDADE ESTADUAL DE CAMPINAS - CENTRO DE BIOLOGIA MOLECULAR E ENGENHARIA GENÉTICA
CIDADE UNIVERSITÁRIA "ZEFERINO VAZ" - BARÃO GERALDO, CX.P.: 6010 - CEP 13083-875 - CAMPINAS - SP - BRASIL
FONE: (55-0xx19) 3521.1147, 3521.1130 ou 3521.1131 - FAX: (55-0xx19) 3788.1089