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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS
TESE DE DOUTORADO

DESENVOLVIMENTO DE MARCADORES DE DNA PARA MAPEAMENTO GENÉTICO E CARACTERIZAÇÃO DA DIVERSIDADE EM FEIJÃO-CAUPI

ALBERTO VINICIUS CASIMIRO ONOFRE

Recife, 2012

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Tese apresentada ao **Programa de Pós-Graduação em Ciências Biológicas** da Universidade Federal de Pernambuco como requisito para obtenção do grau de **Doutor em Ciências Biológicas.**

Orientadora: Profª Drª Ana Maria Benko Iseppon (Depto. de Genética, UFPE)

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As mais altas árvores são oriundas de minúsculas sementes.

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AFLP Polimorfismo de Comprimento de Fragmentos Amplificados (AFLP, do inglês *Amplified Fragment Length Polymorphism*)

BAC Cromossomo Artificial de Bactéria (BAC, do inglês *Bacterial Artificial Chromomossomes*)

BC Retrocruzamento (BC, do inglês *Backcross*)

BICMV Virus Mosaico do Caupi variedade "Blackeye" (BICMV, do inglês *Blackeye cowpea mosaic virus*)

BSA Análise de Segregantes em Grupos (BSA, do inglês *Bulked Segregant Analysis*)

CABMV Vírus do Mosaico do Caupi Transmitido por Afídio (CABMV, do inglês *Cowpea aphid borne mosaic virus*)

CAPS Polimorfismo de Sequência Amplicada e Clivada (CAPS, do inglês *Cleaved Amplified Polymorphic Sequence*)

CBF Comissão Brasileira de Feijão

cDNA DNA complementar (cDNA, do inglês *Complementary DNA*)

cM *CentiMorgans*

CNPAF Centro Nacional de Pesquisa de Arroz e Feijão

CONAB Companhia Nacional de Abastecimento

CPAMN Centro de Pesquisa Agropecuária do Meio-Norte

CPMV Vírus Mosaico do Caupi (CPMV, do inglês *Cowpea Mosaic Virus*)

CPSMV Vírus Mosaico Severo do Caupi (CPSMV, do inglês *Cowpea severe mosaic virus*)

cpSSR (cpSSR, do inglês *Chloroplast Simple Sequence Repeats*)

CTAB (CTAB do inglês *Cetyl-trimethyl-amoniumbromide*)

DAF Impressão Genômica da Amplificação do DNA (DAF, do inglês *DNA Amplification Fingerprinting*)

DarTs Tecnologia de Arranjos de Diversidade (DarTs, do inglês *Diversity Arrays Technology*)

Continuação na próxima página.

Continuação da Lista de Abreviatura.

DH	Linhas Duplo-Haploides (DH, do inglês D*ouble Haploids*)
DNPEA	Departamento Nacional de Pesquisa e Experimentação Agropecuárias
Embrapa	Empresa Brasileira de Pesquisa Agropecuária
EPACE	Empresa de Pesquisa Agropecuária do Ceará
eSSR	Aberviatura de EST-SSR: marcadores SSR derivados de ESTs (eSSR, do inglês *EST derived SSR*)
EST	Seguências Expressas (EST, do inglês *Expressed Sequence Tag*)
FAO	Organização das Nações Unidas para Agricultura e Alimentação (FAO, do inglês *Food and Agriculture Organizations*)
GSR	Transcrito de Sequência do Espaço Gênico (do ingles *Gene-Space Sequence Read*)
GSS	Sequência do Espaço Gênico (GSS, do inglês *Gene Space Sequence*)
He	Herezigosidade Esperada (He, do inglês *Expected Heterozigosity*)
Ho	Herezigosidade Observada (Ho, do inglês *Observed Heterozigosity*)
IITA	Instituto Internacional de Agricultura Tropical (IITA, do inglês *International Institute of Tropical Agriculture*)
IPA	Instituto Agronômico de Pernambuco
ISSR	Região Interna de Seqüências Simples Repetidas (ISSR, do inglês *Inter Simple Sequence Repeats*)
ITS	Espaçador intergénico (ITS, do inglês *Internal Transcribed Spacer*)
LG	Grupo de Ligação (LG, do inglês *Linkage Group*)
LOD	Logaritmo da Probabilidade (LOD, do inglês *Logarithm of an Odds*)
Mb	Mega pares de bases = 1.000.000 bp (bp, do inglês *base pair*)

Continuação na próxima página.

Continuação da Lista de Abreviatura.

MI	Índice do marcador (MI, do inglês *Marker Index*)
PCR	Reação em Cadeia da Polimerase (PCR do inglês *Polymerase Chain Reaction*)
PIC	Conteúdo de Informação Polimórfica (PIC, do inglês *Polymorphism Information Content*)
QTLs	Locos de Caracteres Quantitativos (QTLs, do inglês *Quantitative Trait Loci*)
RAPD	DNA Polimórfico Amplificado ao Acaso (RAPD, do inglês *Randomly Amplified Polymorphic DNA*)
RILs	Linhas Endogâmicas Recombinantes (RILs, do inglês *Recombinant Inbred Lines*)
RP	Poder de Resolução (RP, do inglês *Resolving Power*)
SBMV	Vírus Mosaico do Feijoeiro Sul (SBMV, do inglês *Southern bean mosaic virus*)
SCAR	Sequências Amplificadas de Regiões Caracterizadas (SCAR, do inglês *Sequence Characterized Amplified Region*)
SFP	Polimorfismo de Caráter Único (SFP, do inglês *Single Feature Polymorphism*)
SNP	Polimorfismo de Base Única (SNP, do inglês *Single Nucleotide Polymorphism*)
SSR	Sequências Simples Repetida (SSR, do inglês *Simple Sequence Repeat*)

RESUMO

O feijão-caupi é uma das principais leguminosas cultivadas em todo o mundo. Além da importância econômica e social, essa espécie possui atributos biológicos que a tornam um interessante organismo para a pesquisa em biotecnologia. O uso de marcadores moleculares tem contribuído para os estudos de diversidade dos acessos depositados em bancos de germoplasma e das cultivares mais utilizadas pelos produtores, bem como no desenvolvimento de mapas de ligação. O presente trabalho teve como objetivo saturar o mapa genético, previamente estabelecido, com a população de interesse para o melhoramento da cultura de feijão-caupi no Brasil (BR 14-Mulato x IT85F2687). O mapa de ligação foi construído com base em marcadores SSR (*Simple Sequence Repeats*), ISSR (*Inter Simple Sequence Repeat*), AFLP (*Amplified Fragment Length Polymorphism*) e DAF (*DNA Amplification Fingerprinting*). O mapa de ligação foi construído através do programa MapMaker 2.0. Adotando o valor do limite de detecção (LOD) de 3,0 e uma frequência máxima de recombinação de 0,35, foram mapeadas 216 marcas em onze grupos de ligação cobrindo uma distância de recombinação total de 2064,6 cM. Os grupos de ligação variaram de 68,8 cM (Grupo 11) a 323,3 cM (Grupo 1). A distância entre marcadores adjacentes variou entre 0 e 39,5 cM, com média de 9,7 cM. Os resultados encontrados indicam as bases para o desenvolvimento de mapas específicos saturados para identificação de locos de herança quantitativa de utilidade em programas de melhoramento do feijão-caupi, bem como para estudos comparativos de mapeamento a partir da integração entre espécies do gênero *Vigna*. Esses marcadores representam uma ferramenta útil para isolamento de genes de resistência a estresses bióticos e abióticos desta espécie via clonagem baseada em mapeamento genético.

Palavras-chave: Feijão-caupi, mapa genético, marcador molecular, melhoramento genético.

ABSTRACT

Cowpea is an important legume crop grown worldwide. Besides the economical and social importance of this crop, the species exhibits biological features of an important organism for biotechnological studies. The use of molecular markers has contributed to studies regarding diversity of germplasm bank and cultivars widely used by farmers, as well as to provide tools for development of high-density genetic maps. This work fits in this context, aiming to saturate the first map for a population segregating for important features in Brazil (BR 14-Mulato x IT85F2687) using new molecular markers such as SSR (Simple Sequence Repeats), ISSR (Inter Simple Sequence Repeat), AFLP (Amplified Fragment Length Polymorphism) and DAF (DNA Amplification Fingerprinting). The linkage map was constructed using the Mapmaker 2.0 software. Using a LOD score of 3.0 and a recombination frequency of 0.35, 216 markers were mapped into eleven linkage groups, covering a total recombination distance of 2064.6 cM. The length of each linkage group ranged from 68.8 (LG11) to 323.3 cM (LG1). The distance between adjacent markers varied between 0 and 39.5 cM with average of 9.7 cM. The results obtained point the basis for the development of specific saturated maps for studies in quantitative traits to be used in cowpea programs and in comparison studies with integration of *Vigna* linkage maps. These markers represent an useful tool for genes isolation involved in resistance to biotic and abiotic stresses via map-based cloning.

Key-words: cowpea, genetic map, molecular makers, breeding program.

1. INTRODUÇÃO

O feijão-caupi (*Vigna unguiculata* (L.) Walp.) é uma importante leguminosa cultivada em regiões tropicais e subtropicais, estando presente em 65 países de diferentes continentes, na Ásia e Oceania, sudeste da Europa, África, sudeste dos EUA, América Central e América do Sul (UCHÔA *et al.*, 2007). No Brasil, com destaque para as regiões Norte e Nordeste, o feijão-caupi tem uma grande importância como fonte geradora de emprego e renda e constitui-se em uma das principais fontes de proteína de origem vegetal para o pequeno produtor, sendo de suma importância para a agricultura tradicional e familiar. Em termos de mercado, destaca-se por ser uma cultura bastante versátil, podendo ser comercializada na forma de grãos secos (mercado principal), grãos imaturos (grãos verdes ou frescos), broto de feijão e farinha de acarajé, entre outros. Além disso, a cultura do feijão-caupi é utilizada para adubação verde e na alimentação animal como forragem e ensilagem ou para produção de feno (FREIRE FILHO *et al.*, 2005; ANDRADE *et al.*, 2010).

A cultura, explorada tradicionalmente por pequenos e médios agricultores com baixo nível tecnológico, tem sido pesquisada mais intensamente nas últimas décadas, apresentando melhorias técnicas e econômicas no sistema de produção, fato que contribui para a participação de empresários de médio e grande porte na produção e comercialização do feijão-caupi (BEZERRA *et al.*, 2008).

Estimativas da FAO (2009) indicam que a cultura atingiu 3,6 milhões de toneladas em 12,5 milhões de hectares, produção esta alcançada em 36 países. Neste cenário, a Nigéria é o maior produtor e responde por cerca de 60% do total produzido de feijão-caupi (DEMARCHI, 2008). O Brasil é o terceiro maior produtor, registrando aumento de produção e produtividade nos últimos anos. Apesar do aumento da área plantada, diversos fatores abióticos como seca e salinidade e, fatores bióticos como pragas e doenças, têm determinado oscilações na produção total do Brasil.

Para que avanços consideráveis sejam alcançados no melhoramento desta cultura, é imprescindível obter progressos na compreensão da sua estrutura genômica. Os mapas de ligação fornecem informações básicas da organização do genoma, necessária para a realização de

estudos mais avançados, como o mapeamento de genes de interesse, estes últimos associados às características qualitativas ou quantitativas, bem como os estudos comparativos de sintenia genômica. Permite ainda o estabelecimento de programas de melhoramento com maior grau de previsibilidade de resultados (PEREIRA *et al.*, 2009).

O recente incremento da disponibilidade de marcadores moleculares tornou possível a definição dos grupos de ligação em mapas genéticos em larga escala. Alguns dos marcadores usados no mapeamento são RAPD (DNA polimórfico amplificado ao acaso, do inglês *Randomly Amplified Polymorphic DNA*), AFLP (Polimorfismo de comprimento de fragmentos amplificados, do inglês *Amplified Fragment Length Polymorphism*), SSR (Sequências Simples Repetida, do inglês *Simple Sequence Repeat*), SNP (Polimorfismo de Base Única, do inglês *Single Nucleotide Polymorphism*), DarTs (Tecnologia de Arranjos de Diversidade, do inglês *Diversity Arrays Technology*) e CAPS (Polimorfismo de Sequência Amplicada e Clivada, do inglês *Cleaved Amplified Polymorphic Sequence*) (MOLLINARI *et al.*, 2008). Em espécies de leguminosas de importância econômica, a genômica estrutural tem permitido avanços significativos quanto à localização e organização dos genes (WANG *et al.*, 2008; MORETZSOHN *et al.*, 2009; AGBICODO *et al.*, 2010; GARCIA *et al.*, 2011; SUN *et al.*, 2012).

O primeiro mapa de ligação em cruzamento intraespecífico com feijão-caupi foi reportado por Menéndez *et al.* (1997). Ouédraogo *et al.* (2002), utilizando a população de mapeamento analisada por Menéndez *et al.* (1997) – nesse caso em F_9 – construíram o mais avançado mapa de ligação de referência para o genoma de feijão-caupi, tanto em relação à cobertura do genoma, como em relação à densidade de marcadores. Além do mapa em si, foram identificados marcadores associados a várias características de interesse agronômico, tais como resistência a nematoide das galhas, viroses e fungos. Outros mapas foram desenvolvidos para encontrar marcadores associados a genes de resistência a doenças e pragas de importância em regiões da África (MUCHERO *et al.*, 2009a; MUCHERO *et al.*, 2010).

Recentemente, o primeiro mapa para o feijão-caupi utilizando cultivares adaptadas as condições edafoclimáticas do Brasil, foi publicado a partir de uma população de linhas endogâmicas puras (RILs, do inglês *Recombinant Inbred Lines*) (AMORIM *et al.*, 2009). Foram obtidos 11 grupos de ligação com 39 marcadores, cobrindo 492,1 cM do genoma. Porém, este mapa de ligação ainda não foi suficientemente saturado devido ao baixo número de marcadores

utilizados, o que tem dificultado o trabalho de melhoristas interessados no isolamento de genes de importância agronômica a partir de várias técnicas de clonagem. Assim, a saturação deste mapa preliminar é de grande interesse, pois deve aumentar, por exemplo, a chance de encontrar marcadores associados a genes relacionados à produtividade e resistência a estresse biótico e abiótico e para a melhor compreensão da estrutura genômica e identificação das regiões de interesse.

Nesse contexto, o objetivo principal deste trabalho foi contribuir para a saturação do mapa genético de ligação da população F_6-F_7 resultante do cruzamento entre os acessos BR 14-Mulato e IT85F-2687 descrita por Amorim *et al.* (2009), utilizando marcadores aleatórios DAF (Impressão Genômica da Amplificação do DNA, do inglês *DNA Amplification Fingerprinting*), ISSR (Região Interna de Seqüências Simples Repetidas, do inglês *Inter Simple Sequence Repeats*), SSR genômicos e funcionais e AFLP. Tal mapa será utilizado futuramente na identificação de QTLs (Locos de Caracteres Quantitativos, do inglês *Quantitative Trait Loci*) relacionados à produtividade e resistência a doenças de importância econômica no feijão-caupi.

Esta tese é parte integrante do projeto "Genômica Estrutural, Funcional e Comparativa do feijão-caupi - RENORBIO" e "Mapeamento, Identificação e Validação de genes no Transcriptoma de Feijão-caupi". Uma ampla gama de genes potencialmente úteis ao melhoramento da cultura encontra-se disponível, tendo sido obtida por meio do projeto RENORBIO, tendo gerado cerca de 21 milhões de sequências expressas (transcritos) sob diversas condições, com ênfase para contrastes sob estresse abiótico (salinidade) e sob estresse biótico causado pelo vírus do mosaico severo do feijão-caupi. A proposta pretende propiciar o máximo aproveitamento aos dados obtidos até o momento, mantendo o intercâmbio entre os principais grupos atuantes no estudo e melhoramento do feijão-caupi. Um dos principais objetivos é a identificação e validação de genes candidatos e também de QTLs potencialmente úteis para fins de melhoramento desta cultura a partir da integração de genes validados dos estudos prévios de transcriptoma e estratégias de mapeamento, de modo a propiciar a rápida conversão dos dados gerados em benefício da cultura vegetal em tela. Pretende-se uma imediata aplicação para o melhoramento convencional a partir do mapeamento genético, sendo esta estratégia complementada por inferências biotecnológicas, incluindo cultivo, transformação e regeneração *in vitro* (BENKO-ISEPPON *et al.*, 2010).

2. OBJETIVOS

2.1 OBJETIVO GERAL

Saturar o mapa de ligação previamente estabelecido, com marcadores DAF, ISSR e CAPS, utilizando uma população F_6-F_7 resultante do cruzamento entre os acessos BR 14-Mulato e IT85F-2687, com marcadores moleculares do tipo DAF, SSR, ISSR e AFLP.

2.2 OBJETIVOS ESPECÍFICOS

1. Caracterizar, sob o ponto de vista molecular, acessos de feijão-caupi contrastantes para fenótipos de resistência a estresses bióticos e abióticos, visando identificar marcadores ISSR, DAF, SSR e AFLP promissores para o melhoramento genético;

2. Selecionar marcadores DAF, SSR, ISSR e AFLP informativos nos parentais, proporcionando sua inclusão no mapa;

3. Testar a transferibilidade de marcadores SSR desenvolvidos para *Vigna radiata*, *Vigna angularis* e *Phaseolus vulgaris* em feijão-caupi, selecionando os marcadores transferíveis polimórficos para inclusão no mapa genético;

4. Comparar os resultados do mapa parcial de ligação de feijão-caupi com outros estudos de mapeamento genético no gênero *Vigna*.

3. REVISÃO DA LITERATURA

3.1 CARACTERÍSTICAS TAXONÔMICAS, ORIGEM E DOMESTICAÇÃO DO FEIJÃO-CAUPI

O feijão-caupi é uma espécie cultivada pertencente à ordem Fabales, família Fabaceae, subfamília Faboideae, tribo Phaseoleae, subtribo Phaseolineae, gênero *Vigna*, subgênero *Vigna*, secção *Catiang*, espécie *V. unguiculata* (L.) Walp. e subespécie *unguiculata* (MARÉCHAL *et al.*, 1978; PADULOSI; NG, 1997). A espécie *V. unguiculata*, além da subespécie *unguiculata*, tem duas subespécies cultivadas: *V. unguiculata cilindrica* (L.) Van Eseltine e *V. unguiculata sesquipedalis* Fruw.; e três silvestres: *V. unguiculata dekindtiana* (Harms) Verd., *V. unguiculata tenuis* (E. Mey.) M. M. & S. e *V. unguiculata stenophylla* (Harvey) M. M. & S. (PRATAP, KUMAR; 2011).

As demais espécies mais cultivadas do gênero *Vigna* nos países da África, Ásia e algumas regiões da América Latina pertencem ao subgênero *Ceratotropis*: feijão-mungo-verde [*V. radiata* (L.) Wilczek], feijão-rajado ou feijão-mungo-preto [*V. mungo* (L.) Hepper], feijão-adzuki [*V. angularis* (Willd.) Ohwi & Ohashi], feijão-arroz [*V. umbellata* (Thunb.) Ohwi & Ohashi], além da espécie nativa *V. aconitifolia* (Jacq.) Maréchal, do mesmo subgênero e considerada basal para o grupo (MARÉCHAL *et al.*, 1978; PADULOSI; NG, 1997).

Alguns autores apontam a África como o centro de origem e domesticação do feijão-caupi, uma vez que uma maior concentração de espécies do gênero, bem como do número elevado de espécies endêmicas, foram encontrados nessa região (Ng; MARÉCHAL, 1985; BA *et al.*, 2004). Há outra corrente de pesquisadores que sustentam a hipótese de que a origem da planta seria na Ásia ou América do Sul (FREIRE FILHO, 1988; MAGLOIRE, 2005; SIMON *et al.*, 2007).

No continente americano, acredita-se que a introdução do feijão-caupi tenha ocorrido a partir da Europa e do oeste da África, estando relacionada com a colonização espanhola e com o tráfico de escravos no século XVI. No Brasil, provavelmente, foi introduzida a partir do estado da Bahia, espalhando-se pelos colonizadores para outras áreas da região Nordeste e para outras regiões do país (FREIRE FILHO, 1988). Por outro lado, considerando a diversidade genética do germoplasma da espécie no país, Simon *et al.* (2007) sugeriram que diversos eventos de

introdução devem ter ocorrido, especialmente considerando que escravos de diversas regiões da África foram introduzidos em diferentes regiões e portos brasileiros.

A maior coleção de germoplasma de feijão-caupi, com cerca de 8.000 acessos, está localizada na Nigéria, onde é mantida pelo IITA (Instituto Internacional de Agricultura Tropical, do inglês *International Institute of Tropical Agriculture*). No Brasil, o principal banco ativo de germoplasma é administrado pela Embrapa Meio-Norte ou Centro de Pesquisa Agropecuária do Meio-Norte – CPAMN (Teresina, PI). A Coleção de Base (coleção de ampla abrangência, visando assegurar e atender aos interesses futuros dos programas de melhoramento do feijão-caupi no país) está atualmente constituída de 4.845 acessos do gênero *Vigna*, dos quais, 48% dos acessos são originados de coletas e 52% de introduções (WETZEL; FAIAD, 2001; WETZEL *et al*., 2005).

3.2 ASPECTOS GERAIS E IMPORTÂNCIA ECONÔMICA DO FEIJÃO-CAUPI

Como características básicas do feijão-caupi destacam-se: o ciclo, que pode ser dividido em superprecoce, precoce, médio, médio-precoce, médio-tardio e tardio; a arquitetura da planta, com quatro tipos de portes: ereto, semiereto, semiprostrado e prostrado; o tipo dos grãos, que foi dividido em três classes, sendo: classe branco, classe preto e classe cores (FREIRE FILHO *et al.*, 2005).

O alto valor nutritivo do feijão-caupi é devido à presença nas sementes de grandes quantidades de carboidratos (50-64%), proteínas (20-30% da semente), óleo (0,7-3,5%) e importantes frações de ferro, potássio, fósforo e aminoácidos essenciais como a isoleucina, leucina, fenilalanina, tirosina e metionina (BRESSANI; ELIAS, 1980; PRINYAWIWATKUL *et al.*, 1996; MAIA *et al.*, 2000). Além disso, é um elemento essencial nas regiões semiáridas dos trópicos visto que: (a) apresenta considerável tolerância à seca e ao calor, (b) proporciona uma excelente cobertura vegetal e (c) ajuda a evitar a erosão e contribui para a melhoria da fertilidade de solos em áreas marginais, ao fornecer matéria e resíduos orgânicos que se incorporam ao substrato após a colheita de seus grãos, melhorando a estrutura edáfica, aumentando a disponibilidade de fósforo e contribuindo para a fixação de nitrogênio, pela associação com bactérias fixadoras de N_2 atmosférico (VALENZUELA; SMITH, 2002).

Embora a importância desta cultura seja reconhecida para as populações de regiões semiáridas do Brasil, o seu melhoramento com métodos convencionais de cultivo e seleção vem se mostrando limitado para o desenvolvimento de novas cultivares com resistência múltipla a estresses bióticos e abióticos. Para Oliveira *et al.* (2002), no Brasil existem cultivares com boa aceitação comercial. No entanto, o feijão-caupi ainda apresenta baixos patamares de produtividade (366 kg ha^{-1}) e a produção corresponde a apenas 15% da produção total de feijões (DAMASCENO-SILVA, 2009; LEITE *et al.*, 2009), destacando-se o cultivo em ambientes que apresentam restrições edafoclimáticas e a ocorrência de pragas e doenças como principais fatores determinantes da baixa produtividade e produção.

A região Nordeste brasileira responde por 1.205.839 ha (94,40%) da área e 412.458 toneladas (87,73%) da produção total de feijão-caupi (DAMASCENO SILVA, 2008). A cultura se encontra em expansão no Sudeste e na região Centro-Oeste, sobretudo, no estado do Mato Grosso, onde a produtividade pode ultrapassar uma tonelada por hectare e – devido à sua rusticidade e ciclo curto – pode se constituir em importante espécie para o cultivo durante o período de outono-inverno, tornando-se importante componente nos sistemas de rotação de culturas para o sistema de plantio direto (DURANTE; CORREA, 2011).

O consumo médio de feijão-caupi no Brasil é de 20 kg/ano por pessoa, e estima-se que a cultura gere 2,4 milhões de empregos e que abasteça a mesa de 27,5 milhões de nordestinos. Esses dados são extremamente importantes, pois refletem a participação da cultura no contexto de geração de emprego, de renda e da produção de alimentos no país, o que a credencia para receber maior atenção por parte das políticas de abastecimento e por parte dos órgãos de apoio à pesquisa (FREIRE FILHO *et al.*, 1999; SANTOS *et al.*, 2007).

O mercado do feijão-caupi caracteriza-se pela volatilidade de preços. Mesmo com as mudanças ocorridas na sua estrutura de produção (com impacto direto na produtividade da cultura) a oferta é sujeita a variações determinadas, também, pelas condições climáticas e fitossanitárias (AGUIAR; SANTOS, 2011).

Estima-se que nas regiões Norte e Nordeste exista um déficit permanente de oferta de feijão-caupi, respectivamente de 17.576,7 e 102.281,3 toneladas. Já na região Centro-Oeste, onde o cultivo do feijão-caupi ainda está se expandindo, há um superávit de 38.271,7 toneladas. Além disso, o número de cultivares melhoradas de feijão-caupi é muito pequeno quando

comparado ao de outras culturas anuais cultivadas no país. Desse modo, com essa pequena oferta de cultivares e considerando que muitas já deixaram de ser cultivadas, as cultivares melhoradas ocupam uma pequena parte da área plantada com feijão-caupi no Brasil. Diante dessa realidade, tem-se a perspectiva de ampliar a rede de melhoramento genético de feijão-caupi para todas as regiões (FREIRE FILHO *et al.*, 2011).

3.3 MELHORAMENTO DO FEIJÃO-CAUPI

O melhoramento genético convencional do feijão-caupi começou na década de 60. O principal programa brasileiro de melhoramento de feijão-caupi no Brasil está localizado em Terezina, PI e é coordenado pela Embrapa Meio-Norte, tendo como principais objetivos: (a) o aumento da produtividade e melhoria da qualidade visual e nutricional dos grãos; (b) o aumento da adaptabilidade, a estabilidade e tolerância aos estresses hídricos e salinos; (c) a incorporação de resistência múltipla a doenças e pragas e (d) a obtenção de porte mais compacto e ereto, possibilitando sua colheita mecânica e seu processamento em escala industrial (FREIRE FILHO *et al.*, 1999).

Vale salientar que trabalhos pioneiros também foram desenvolvidos em alguns programas de melhoramento como aqueles do Departamento Nacional de Pesquisa e Experimentação Agropecuárias (DNPEA) da Comissão Brasileira de Feijão (CBF), Centro Nacional de Pesquisa de Arroz e Feijão (CNPAF) e em órgãos estaduais, como o Instituto Agronômico de Pernambuco (IPA) e Empresa de Pesquisa Agropecuária do Ceará (EPACE) (QUEIROZ; LOPES, 2007; FREIRE FILHO *et al.*, 2011).

As principais doenças e pragas que ocorrem no feijão-caupi podem causar perdas superiores a 50% na produção de vagens, quando medidas de controle adequadas não são utilizadas. A partir da década de 80 foram desenvolvidas as primeiras cultivares com resistência múltipla a viroses, bacterioses e doenças fúngicas, obtidas dos cruzamentos entre cultivares locais e cultivares introduzidas. A primeira foi a cultivar BR1-Poty, produtiva e altamente resistente aos vírus CPSMV (vírus do mosaico severo do feijão-caupi, do inglês *Cowpea severe mosaic virus*) e CABMV (vírus do mosaico do feijão-caupi transmitido por afídeo, do inglês *Cowpea aphid borne mosaic virus*). A partir da cultivar BR1-Poty, foram lançadas outras cultivares para as diferentes regiões: as cultivares EPACE 10, EPACE 11 e Patativa no Ceará; as

cultivares IPA-204, IPA-205, e IPA-206, em Pernambuco; enquanto no Piauí destacam-se as cultivares BR-10 Piauí, BR-14 Mulato, BR-17 Gurguéia, BRS-Marataoã e BRS-Guariba (BENVINDO, 2007).

Embora muitas cultivares com resistência múltipla a viroses tenham sido lançadas, os programas de melhoramento não têm sido um processo contínuo, ou seja, a resistência não é incorporada nas cultivares lançadas mais recentemente. Isto ocorre devido ao fato de que os genes identificados nem sempre apresentam um amplo espectro de ação (ASSUNÇÃO *et al.*, 2005).

Quando a infecção do vírus do mosaico severo ocorre em plantas jovens, os sintomas são drásticos e visíveis em todas as partes aéreas da planta, causando inclusive necrose da extremidade superior do caule, morte dos brotos terminais e queda prematura das folhas. Nas folhas, os sintomas manifestam-se na forma de manchas cloróticas e necróticas, mosaico severo, distorção foliar, redução da lâmina foliar, bolhosidade, clareamento das nervuras, necrose sistêmica e morte de algumas cultivares. Em muitas cultivares, podem-se observar manchas irregulares nas vagens e nas sementes, sendo que estas, normalmente, apresentam-se chochas, com baixa taxa de germinação (LIMA *et al*., 2005). Em função disso, os danos na produção são bastante significativos, dependendo da cultivar envolvida e da época de inoculação.

Medidas de prevenção, geralmente envolvem a aplicação semanal de inseticidas para controlar a população de vetores e consequentemente a disseminação do vírus (COSTA *et al.*, 1978), um método prejudicial ao meio ambiente que infelizmente não é efetivo na estação chuvosa, quando as plantações estão em fase de crescimento (UMAHARAN *et al*., 1996; PAZ *et al.*, 1999). O alto custo também tem desencorajado a adoção do controle químico dos vetores pelos agricultores. Por conta da ampla distribuição do vírus nas regiões de plantio e dos diferentes tipos de hospedeiros naturais existentes no Brasil, a identificação de fontes de resistência está entre as maiores preocupações dos pesquisadores que trabalham com o feijão-caupi (FREIRE-FILHO *et al.*, 1999).

Além das viroses, o complexo de patógenos do solo que envolve os gêneros *Rhizoctonia*, *Fusarium*, *Macrophomina*, *Sclerotium* e *Meloidogyne*, vem crescendo de importância, por isso, maior atenção no que se refere à identificação de fontes de resistência e desenvolvimento de cultivares resistentes (FREIRE FILHO *et al.*, 2005; SOBRINHO *et al.*, 2005). Os nematoides-

das-galhas, principalmente *Meloidogyne incognita* e *Meloidogyne javanica*, possuem extensa disseminação em toda área de cultivo do feijão-caupi e são os principais responsáveis pela redução de produção. Já as espécies de insetos *Callosobruchus maculatus* e *Callosobruchus chinensis*, pertencentes à família Bruchidae, podem atacar as sementes de feijão-caupi ainda em campo e causar perdas dos grãos armazenados estimadas em 20–60% (ABROL, 1999; TARVER *et al.*, 2007).

Diversos fatores ambientais também podem afetar o desempenho do feijão-caupi, tais como temperaturas muito baixas ou altas, bem como seca, salinidade e falta ou excesso de elementos nutritivos (NASCIMENTO, 2009).

Como a resposta ao estresse salino e hídrico é de natureza poligênica, o melhoramento não pode ser tratado como um problema de fácil solução. Com isso, não será apenas extrapolando resultados positivos a partir de estudos em casa de vegetação e/ou campo, transferência ou superexpressão de um ou poucos genes que serão desenvolvidas plantas produtivas tolerantes à salinidade. Pelo contrário, um problema com tal complexidade só poderá ser enfrentado com o uso de diferentes metodologias associadas a diferentes áreas como a fisiologia, genômica, proteômica, genética e biologia molecular, entre outras, uma vez que o estresse abiótico envolve um conhecimento multidisciplinar (HONG-BO *et al.*, 2006).

Visando a obtenção de cultivares com maior estabilidade de produção para cultivo nas diferentes épocas de semeadura, condições ambientais e resistentes às principais doenças e pragas que acometem o feijão-caupi, a utilização de genitores divergentes que apresentem alelos de interesse em cruzamentos pode ser uma opção vantajosa (CHIORATO, 2004).

A divergência genética entre genitores pode promover combinações gênicas favoráveis, mediante efeitos de aditividade, pleiotropia e epistasia. A caracterização genética de bancos de germoplasma permite o conhecimento da variabilidade existente na espécie, orientando o trabalho do melhorista no planejamento de cruzamentos (CHIORATO, 2005).

3.4 MARCADORES MOLECULARES E BIOTECNOLOGIA DO FEIJÃO-CAUPI

Além dos aspectos socioeconômicos, o feijão-caupi apresenta uma série de características, a exemplo do pequeno tamanho do genoma estimado em 630 Mb e o modo preferencial de reprodução por autogamia, o que o credencia como uma planta modelo em leguminosas (XU *et al*., 2009; POTTORFF *et al*., 2012). Estudos sobre a organização de sequências e genes no genoma do feijão-caupi podem oferecer a base para interpretação genética e molecular que auxiliam na seleção e transferência de genes agronomicamente importantes em programas de melhoramento convencional ou a partir da Biotecnologia via transformação genética (transgenia).

O emprego de técnicas de cultura de tecidos é um pré-requisito para transformação genética. Em feijão-caupi vários protocolos de regeneração *in vitro* foram estabelecidos e a transformação genética pode ser bem sucedida a partir de co-cultivos da bactéria *Agrobacterium tumefaciens* e por meio da eletroporação de embriões (AZEVEDO *et al.*, 2007; IVO *et al.*, 2008; AASIM *et al.*, 2010; BAKSHI *et al.*, 2011). A principal contribuição desta tecnologia em feijão-caupi está no desenvolvimento de cultivares resistentes a insetos (OBEMBE, 2009). No entanto, outros genes de interesse apresentam potencial de transferência via engenharia genética. A expectativa é que com o uso dessa técnica no melhoramento genético ocorra um aumento na produção, obtenção de cultivares mais tolerantes aos estresses bióticos e abióticos, aumento no valor nutricional e desenvolvimento de alternativas para indústrias como as de combustíveis e farmacêuticas (MONQUERO, 2005). Mas tanto para o melhoramento convencional como para a transgenia, é necessário identificar os genes que controlam as características de interesse agronômico.

A tecnologia de marcadores moleculares, aliada às técnicas clássicas do melhoramento, pode contribuir significativamente para o conhecimento básico da cultura e do caráter estudado e para a geração e o desenvolvimento de produtos melhorados (CAIXETA *et al.*, 2003).

Estudos com marcadores moleculares têm revelado polimorfismos genéticos importantes na caracterização de feijão-caupi. Tais estudos têm contribuído para a caracterização de germoplasma, programas de melhoramento genético e entendimento das relações filogenéticas no gênero *Vigna* (SIMON *et al.*, 2007; ONOFRE, 2008; SPIAGGIA *et al*., 2009; SAWADOGO *et al.*, 2010; VIJAYKUMAR *et al.*, 2010). A detecção de polimorfismos de DNA por meio de

marcadores pode ser feita de diferentes formas. Marcadores como RAPD, SSR e AFLP são alguns dos mais utilizados em feijão-caupi.

O marcador molecular DAF é similar à técnica RAPD, com pequenas variações no comprimento e concentração do *primer* usado na amplificação mediada pela DNA polimerase, e na separação e detecção dos fragmentos. A estratégia básica envolve a amplificação de fragmentos ao acaso do DNA genômico, com um único *primer* de sequência arbitrária. Cada *primer* arbitrário de 8-10 bp pode parear em diversos pontos no genoma, resultando, assim, em vários fragmentos ou bandas que são separados de acordo com o tamanho por eletroforese em gel de agarose ou acrilamida. A ligação do *primer* ao DNA é facilitada pela baixa temperatura de anelamento. O polimorfismo genético observado ocorre devido a diferenças no DNA (deleções ou inserções de nucleotídeos), nos sítios de anelamento ou entre eles. O polimorfismo detectado por marcadores DAF tem natureza binária, isto é, o segmento amplificado está presente ou ausente. Desta forma, estes marcadores não distinguem indivíduo homozigoto dominante de heterozigoto para um determinado lócus (CAETANO-ANOLLÉS; BASSAM, 1993; PEAKALL, 1997).

O interesse em utilizar marcadores DAF ou RAPD está no baixo custo da técnica e por não exigir um conhecimento prévio do genoma. Assim, um mesmo conjunto de *primers* pode ser usado para amplificar o DNA dos mais diversos tipos de plantas. Há empresas que comercializam *kits* de *primers* contendo mais de 1.000 diferentes tipos de sequência (ALZATE-MARIN, *et al.*, 2005). Além disso, a técnica possui as vantagens de ser altamente reprodutível, apresentar herança mendeliana e possuir um bom nível de polimorfismo (CAETANO-ANOLLÉS *et al.*, 1991). Essa técnica tem sido aplicada com êxito na caracterização genética e desenvolvimento de mapas de diversas leguminosas como o grão-de-bico (*Cicer arietinum* L.), soja (*Glycine max* L.), e amendoim (*Arachis hypogaea* L.) (CAETANO-ANOLLÉS *et al.*, 1991; JIANG; PRABHU; GRESSHOFF, 1994; JIANG; GRESSHOFF, 1997; WINTER *et al.*, 2000; BENKO-ISEPPON *et al.*, 2003;), revelando-se relativamente vantajosa quando comparada com o RAPD, notadamente por sua elevada reprodutibilidade e por gerar um grande número de polimorfismo (BENKO-ISEPPON *et al.*, 2003).

Xavier *et al.* (2005), ao avaliarem o polimorfismo em feijão-caupi com marcadores RAPD, encontraram oito iniciadores polimórficos, a partir de 20 testados. Todos os acessos avaliados apresentaram uma similaridade acima de 80%, mesmo quando oriundos de diferentes

áreas geográficas (Brasil, Nigéria e EUA). Os autores sugeriram uma limitação de base genética devido ao agrupamento dos acessos de variedades locais brasileiras em apenas um grupo. Por outro lado, Simon *et al.* (2007) mostraram que *primers* DAF identificaram com maior precisão acessos de feijão-caupi. Apesar da base genética estreita indicada por outros autores, que caracteriza as coleções de feijão-caupi encontradas no Brasil, a variabilidade encontrada por esses autores foi relativamente alta.

A técnica de SSR baseia-se no uso de pares de *primers* na reação de PCR (Reação em Cadeia da Polimerase, do inglês *Polymerase Chain Reaction*) para detectar variação em locos de sequência repetitiva. Estas são constituídas de um a seis nucleotídeos que se repetem lado a lado (por exemplo CTCTCTCTCTCT), no genoma de plantas e animais (BUSO *et al.*, 2003).

Os microssatélites podem ser caracterizados de acordo com sua repetição e motivos. Com relação à repetição, essas sequências são classificadas em perfeitas, imperfeitas e compostas. Os microssatélites perfeitos são constituídos por extensões ininterruptas de um motivo particular. Por outro lado, as SSRs imperfeitas consistem em repetições interrompidas, ao longo de sua extensão, por nucleotídeos que não se encaixam na estrutura repetitiva ou motivo original. No caso dos microssatélites compostos observam-se dois ou mais grupos de motivos diferentes adjacentes (Figura 1). Já em relação ao motivo, os SSR podem ser classificados em dinucleotídeos (GA_n), trinucleotídeos (TAG_n), tetranucleotídeos ($CAGT_n$), pentanucleotídeos ($GTAAC_n$) e hexanucleotídeos ($GTAACG_n$) (DEPEIGES *et al.*, 1995; OLIVEIRA *et al.*, 2006).

Embora as sequências microssatélites geralmente variem de um indivíduo para outro, as sequências de DNA que as flanqueiam são muito conservadas entre indivíduos da mesma espécie, o que proporciona o desenho de *primers* específicos para essas regiões adjacentes às sequências microssatélites e a transferibilidade de primers entre espécies diferentes. Os produtos da amplificação podem ser visualizados em gel de poliacrilamida desnaturante ou não desnaturante, em gel de agarose de alta resolução ou por meio de *primers* fluorescentes em sequenciador automático, sendo os polimorfismos entre bandas decorrentes dos tamanhos diferentes de elementos simples repetidos (SOUZA, 2001; BORÉM; CAIXETA, 2006).

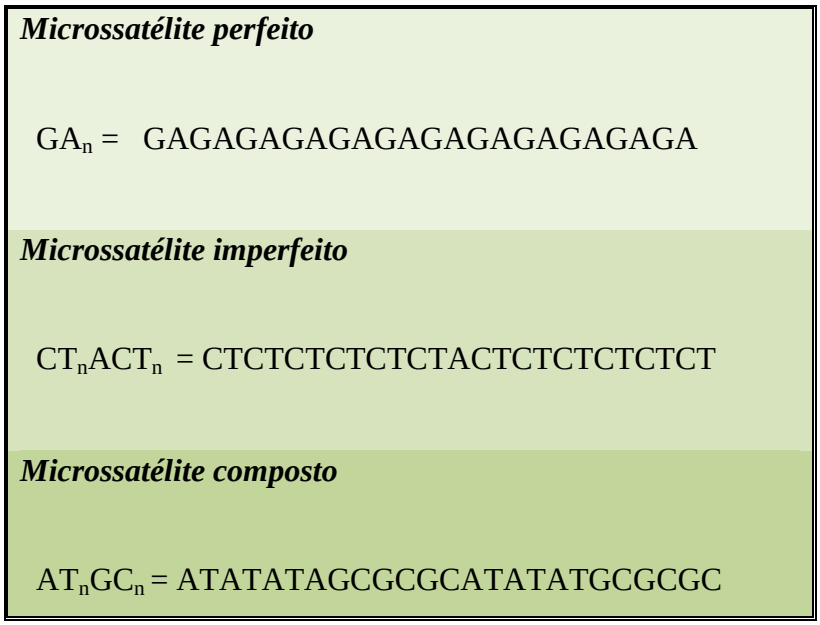

Figura 1. Classificação dos microssatélites de acordo com a estrutura das unidades de repetições. Adaptado de Bortoleti (2012).

Segundo Buso *et al.* (2003) os marcadores microssatélites podem ser utilizados com sucesso por melhoristas vegetais, pois possuem várias vantagens, como: a) natureza codominante e multialélica; b) alto índice de polimorfismo por se tratarem de sequências com alta taxa evolutiva, o que permite discriminações precisas, mesmo de indivíduos altamente relacionados; c) são abundantes e uniformemente dispersos em todo o genoma de plantas; d) a informação do marcador, baseada nas sequências dos iniciadores, pode ser facilmente publicada e trocada entre laboratórios, melhorando os esforços cooperativos em pesquisa e desenvolvimento.

Os marcadores SSR têm sido amplamente utilizados para caracterização de germoplasma do feijão-caupi (BADIANE *et al.*, 2012) e de outras espécies do gênero *Vigna*, incluindo *V. radiata* (SANGIRI et al., 2007; GWAG *et al.*, 2010), *V. angularis* (XU *et al.*, 2008), *V. subterranea* (SOMTA *et al.*, 2011) e *V. mungo* (BINYAMIN *et al.*, 2011).

Os SSR podem ser derivados de bibliotecas genômicas, denominados de SSRs genômicos, ou de banco de dados de sequências expressas, denominados de EST-SSRs (*Expressed Sequence Tags - Simple Sequence Repeats*), ou ainda de sequências cloroplastidiais (cpSSR - *Chloroplast Simple Sequence Repeats*), apresentando, cada uma delas, diferentes aplicações (SHIRASAWA *et al.*, 2011).

O desenvolvimento de SSR genômicos pode ser feito a partir da construção de bibliotecas ou por técnicas de enriquecimento (ZANE *et al.*, 2002). Segundo Ferreira & Grattapaglia (1998) a desvantagem da técnica é que requer grande quantidade de trabalho no laboratório, exigindo algum conhecimento prévio do genoma da espécie sob estudo, técnicos especializados com domínio de técnicas de biologia molecular e de genética molecular, equipamentos para sequenciamento automático e alocação de recursos financeiros. Apesar disso, o elevado custo é recompensado pelo potencial de informações para pesquisas e desenvolvimento que se abrem ao se possuir tal tecnologia para uma determinada espécie.

Os EST-SSRs são desenvolvidos por meio de análise bioinformática (mineração) de sequências transcritas do genoma depositadas em bancos de dados. Esta estratégia é considerada simples, rápida e econômica, comparado ao SSRs obtidos das bibliotecas de DNA genômico (ZANE *et al.*, 2002). Ao mapear estes microssatélites é possível identificar potenciais candidatos para funções importantes devido a sua homologia a genes envolvidos em diferentes vias metabólicas das plantas e a co-localização com QTLs. As marcas ligadas aos QTLs podem ser utilizadas em seleção assistida por marcadores ou como guias visando à clonagem posicional dos genes ou regiões regulatórias envolvidas no controle do caráter (SENA, 2009). Estes marcadores são desenvolvidos facilmente para espécies com genomas sequenciados. Em leguminosas, EST-SSR foram desenvolvidos para feijão-comum (*Phaseolus vulgaris* L.), ervilha (*Pisum sativum* L.), grão-de-bico (*Cicer arietinum* L.), soja (*Glycine max* L.), amendoim (*Arachis hypogaea* L.) e alfafa (*Medicago sativa*) (EUJAYL *et al.*, 2004; CHOUDHARY *et al.*, 2009; LI *et al.*, 2010; SONG *et al.*, 2010; GARCIA *et al.*, 2011; MISHRA *et al.*, 2012). Devido a maior conservação nas sequências EST-SSRs, estes marcadores são bastante utilizados em estudos de transferibilidade. Esse processo faz com que os marcadores SSRs apresentem um grande potencial no mapeamento comparativo (YU *et al.*, 2004).

Em feijão-caupi, Li *et al.* (2001) detectaram marcadores do tipo microssatélite utilizando 44 pares de *primers* desenvolvidos a partir de bibliotecas enriquecidas. Os autores apontaram uma baixa variabilidade genética nos acessos analisados. Mais recentemente, a partir de base de dados de domínio público, Gupta e Gopalakrishna (2010) desenvolveram marcadores SSR e verificaram em 20 acessos um valor do PIC (conteúdo de informação polimórfica, do inglês *Polymorphism Information Content*) médio de 0,53, sendo considerado informativo. Xu *et al.* (2010) desenvolveram 600 marcadores genômicos derivados de sequências depositadas no *Cowpea Gene Space. Sequence, GSS – Knowledge Base* e 410 derivados de sequências EST. Os

autores analisaram transferibilidade para a subespécie *V. unguiculata sesquipedalis* e a separação genética entre as duas subespécies. Recentemente, estes marcadores foram mapeados em *V. unguiculata* ssp. *sesquipedalis* e podem ser utilizados em estudos comparativos com outras espécies do gênero *Vigna* (XU *et al.*, 2011).

Correia *et al.* (2008) identificaram motivos SSRs em ESTs do projeto NordEST/Renorbio a partir de 4.919 sequências, gerando oito marcadores polimórficos. Segundo os autores, este trabalho ratifica que o conhecimento da ocorrência e frequência de sequências repetidas nos genomas é importante não apenas para um entendimento de sua distribuição, mas, também, para direcionar o desenvolvimento de marcadores SSR específicos para uso em análises genéticas.

O uso de marcadores SSR e a disponibilidade de um mapa de referência de feijão-caupi no Brasil ajudarão a entender a estrutura, função e evolução do genoma dessa cultura, bem como no desenvolvimento de outros mapas genéticos, pela transferência de informação da ordem e ligamento dos marcadores, o que permite uma análise comparativa e integrações de mapas.

3.5 DESENVOLVIMENTO DE MAPAS GENÉTICOS

Um mapa genético é essencialmente um arranjo linear de marcadores, ordenados a partir de valores de recombinação (SALGADO *et al.*, 2011). A metodologia de construção de um mapa integra um grande número de técnicas. Entre estas, destaca-se o desenvolvimento de linhagens progenitoras e populações segregantes adequadas à identificação dos genótipos nos locos marcadores por meio de técnicas de biologia molecular, e a utilização de diversas análises estatísticas e computacionais para a estimativa de ligação e distância entre marcadores (GRATTAPAGLIA; FERREIRA, 2009).

Em mapas genéticos, os marcadores moleculares são informativos quando são polimórficos nos parentais contrastantes, pois dessa forma permitem acompanhar a segregação de alelos que possuem efeito fenotípico de interesse agronômico. Um grande número de marcadores genéticos polimórficos leva à saturação de mapas, o que permite uma eficiente identificação de marcadores ligados a QTLs e contribuições relevantes nos programas de seleção assistida (MORETZSOHN, 2006).

Para identificação de marcadores polimórficos é necessário testar centenas de marcadores nos parentais da população a ser utilizada. A seleção de qual marcador utilizar vai depender de suas características, como por exemplo, vantagens e desvantagens, disponibilidade de marcadores para a espécie, custos, entre outros (MORETZSHON, 2006).

Novas tecnologias de marcadores estão sendo desenvolvidas para identificar o maior número de marcas polimórficas. Marcadores baseados em técnicas de hibridização por microarranjo de DNA (*DNA microarray*), como o DArT e os SFPs (Polimorfismos de Caráter Único, do inglês *Single Feature Polymorphisms*), estão produzindo dados em larga escala e com custo relativamente baixo. A combinação de marcadores SSR e DArTs em grão-de-bico permitiu a geração de um mapa genético com alto grau de saturação (THUDI *et al.*, 2011). Das *et al.* (2008) desenharam marcadores SFP para feijão-caupi baseados em genes de soja. Os autores sugerem que estes marcadores possibilitam uma série de pesquisas relacionadas ao entendimento da herança e identificação de regiões genômicas associadas à resistência/tolerância a estresses bióticos e abióticos.

As populações de mapeamento geralmente utilizadas em plantas são as obtidas por retrocruzamento (BC, do inglês *backcross*), populações F_2, populações F_1 de progenitores heterozigóticos, linhas endogâmicas recombinantes (RILs, do inglês *Recombinant Inbred Lines*) e linhas duplo-haploides (DH, do inglês *Double Haploids*). As RILs são descendentes homozigóticas resultantes de rodadas sucessivas de autofecundação compreendendo as gerações F_2 até F_6. Devido à elevada taxa de homozigose alcançada nas seis gerações, o nível de recombinação é mais alto do que uma população F_2, por exemplo. Além disso, nas populações RILs o mesmo genótipo pode ser avaliado em locais e programas de melhoramentos diferentes, o que possibilita o intercâmbio entre informações genéticas geradas por diferentes grupos de pesquisa (DARVASI; SOLLER, 1995; FERREIRA; GRATTAPAGLIA, 1998; SOARES, 2000).

Para demonstrar que dois locos estão ligados, é necessário definir a frequência máxima de recombinação e o LOD *score* mínimo. Por exemplo, um LOD igual a 3 indica que a ocorrência de ligação é mil vezes mais provável que a de segregação independente. Para separar os grupos de ligação, realizaram-se testes considerando dois locos de cada vez, testes para detectar a ocorrência de desequilíbrio de ligação e o teste do qui-quadrado (χ^2) (BEARZOTI, 2000).

Existem várias ferramentas de software para construção de mapas genéticos. Cheema e Dicks (2009) apresentam 11 ferramentas em ordem cronológica de criação, informando a disponibilidade e aplicação de cada *software*. Segundo Salgado *et al.* (2011), os programas MapMaker, Linkage-1, GQMOL e JoinMap são os mais utilizados para análises de mapas genéticos baseados em marcadores moleculares.

3.6 MAPEAMENTO GENÉTICO NO GÊNERO *VIGNA*

Os mapas genéticos desenvolvidos para espécies do gênero *Vigna* podem ser classificados em dois grupos distintos: mapas de referência, com o objetivo de servir de base para pesquisas futuras (OUÉDRAOGO *et al.*, 2002; HAN *et al.* 2005; CHAITIENG *et al.*, 2006), e mapas específicos, que visam localizar genes de interesse, como genes de resistência a doenças e pragas.

Os primeiros mapas genéticos no gênero *Vigna* foram baseados em marcadores RAPD e RFLP. Kaga *et al.* (1996) utilizaram uma população F_2 resultante do cruzamento interespecífico de *V. angularis* com seu parente silvestre (*V. nakashimae* (Ohwi) Ohwi & Ohasshi). No tota, 132 marcadores foram mapeados, sendo 108 RAPDs, 19 RFLPs e cinco marcadores morfológicos, em 14 grupos de ligação, cobrindo 1.250 cM do genoma.

O primeiro cruzamento intraespecífico de feijão-caupi usado na geração de um mapa genético foi desenvolvido por Menéndez *et al.* (1997) a partir do cruzamento entre duas linhagens melhoradas (IT84S-204 e 524B). Os autores usaram as metodologias de RAPD, RFLP e AFLP para a saturação do mapa, observando que os marcadores gerados eram na sua maioria monomórficos. Foram obtidos 133 marcadores RAPD, 19 RFLPs, 25 AFLPs, três marcadores morfológicos e um enzimático, os quais formaram 12 grupos de ligação e cobriram uma região de 972 cM, valor que indicava um baixo nível de saturação, motivo pelo qual Ouédraogo *et al.* (2002) iniciaram a inclusão de novos marcadores neste mapa. A cobertura do genoma passou para 2.670 cM, com uma distância média de 6,43 cM entre marcadores, tornando-se o mapa de ligação de referência para o genoma de feijão-caupi, tanto em relação à cobertura do genoma, como em relação à densidade de marcadores. Além do mapa em si, foram identificados marcadores associados a várias características de interesse agronômico, tais como resistência a duas raças do parasita *Striga gesnerioides*, à murcha de fusário (*Fusarium oxysporum f. sp.*

tracheiphylum (E.F Smith.) W.C. Snyd & H.N. Hansen), ao nematoide *Meloidogyne javanica* e resistência a viroses *Cowpea mosaic virus* (CPMV), *Cowpea severe mosaic virus* (CPSMV), *Blackeye cowpea mosaic virus* (BICMV), *Southern bean mosaic virus* (SBMV). Vale salientar, no entanto, esses mapas genéticos atendem a demandas dos programas de melhoramento dos Estados Unidos e da África, sendo necessária a associação de marcadores moleculares desenvolvidos a partir de cruzamentos com cultivares brasileiras de feijão-caupi (BENKO-ISEPPON, 2001).

Chaitieng *et al*. (2006) desenvolveram o primeiro mapa de ligação para *V. mungo* com 148 marcadores, comprimento total de 783 cM em 11 grupos de ligação. Os marcadores formaram 11 grupos de ligação com uma distância média entre os marcadores de 5,7 cM, totalizando 768 cM. Por sua vez, o mapa de ligação construído, quando comparado com o mapa de *V. angularis* (HAN *et al*., 2005), apontou para uma alta conservação na ligação entre os locos no que se refere à ordem dos marcadores SSR.

Muchero *et al.* (2009b) publicaram o primeiro mapa de ligação em feijão-caupi com a tecnologia de SNP (Polimorfismo de nucleotídeo único, do inglês *Single Nucleotide Polymorphism*) utilizando seis populações. Foram mapeados 928 SNPs perfazendo um comprimento total de 680 cM em 11 grupos de ligação, com uma distância de 0,73 cM entre os marcadores.

Um recente mapa para subespécie *V. unguiculata* ssp. *sesquipedalis* demonstra o potencial dos marcadores SSR desenvolvidos a partir de banco de dados para feijão-caupi (XU *et al.*, 2011).

Em relação aos estudos direcionados para identificação de marcadores ligados a genes que controlam características de resistência a doenças e pragas em feijão-caupi, Myers *et al.* (1996) utilizaram uma população de mapeamento derivada do cruzamento entre duas cultivares de feijão-caupi contrastantes para característica de resistência ao pulgão *Aphis craccivora* (Koch). A partir da técnica de RFLP foi possível identificar um marcador (bg4D9b) fortemente ligado ao gene de resistência *Rac 1*, e vários marcadores flanqueando esse gene no mesmo grupo de ligação. A forte ligação entre o marcador e o gene levou os autores a concluir que a clonagem do referido gene pode ser feita com sucesso e que a seleção assistida por marcadores pode ser empregada para o desenvolvimento de cultivares resistentes ao pulgão.

Ouédraogo *et al.* (2001) utilizaram os marcadores AFLPs associados ao método de BSA (Análise de Segregantes em Grupos, do inglês *Bulked Segregant Analysis*) para identificar marcadores ligados aos genes *Rsg*2-1 e *Rsg*4-3, que conferem resistência a duas raças de *S. gesneriodes*, em dois cruzamentos com parentais contrastantes. Os autores identificaram marcas associadas no grupo de ligação 1 e também concluíram que é possível utilizar essas informações na seleção assistida por marcadores e eventual clonagem para caracterização do gene que confere resistência a *S. gesneriodes.*

Acerca do mapeamento de caracteres controlados por vários genes de caráter quantitativo, Muchero *et al.* (2010) mapearam três locos associados à resistência às pragas *Thrips tabaci* e *Frankliniela schultzei* usando linhas endogâmicas recombinantes RILs em feijão-caupi. Os QTLs foram identificados nos grupos de ligação 5 e 7, contabilizando entre 9,1 e 32,1% de variância fenotípica.

Muchero *et al.* (2011) analisaram QTLs associados à resistência a *Macrophomina phaseolina* e à maturação do feijão-caupi, a partir da metodologia de SNPs e exploraram a sintenia entre os genes de soja e *Medicago truncatula*. Para o caráter de resistência a *M. phaseolina*, estes autores identificaram oito QTLs (*Mac1 – Mac8*) distribuídos em quatro grupos de ligação. Efeito da maturação sobre a expressão da resistência foram verificados pela colocalização dos QTLs *Mat-2* e *Mat-7*. Os resultados revelaram microssintenia entre feijão-caupi, soja e *M. truncatula*. O grupo de ligação 5 e 7 de feijão-caupi abrange muitas regiões dos cromossomos 2, 14 e 17 de soja e do cromossomo 5 de *M. truncatula*.

Depois de décadas de pesquisas em feijão-caupi, pode-se observar uma quantidade significativa de dados na área genômica. O desenvolvimento de mapas genéticos em programas de melhoramento dos Estados Unidos e da África permitiu a localização de marcadores moleculares associados à resistência a estresses bióticos e abióticos, bem como à maturação e precocidade. Os avanços na área de transformação genética apontaram novas oportunidades para estudos de expressão de genes importantes em vias metabólicas. Além disso, análises de transcriptômica mostraram que muitos genes são expressos quando o feijão-caupi é submetido ao estresse hídrico ou em condições de deficiência de nitrogênio. A proteômica e a metabolômica revelaram as frações proteicas e metabólitos a partir de células embriogênicas em suspensão e em condições de toxicidade de manganês, respectivamente. O mais importante é que os

pesquisadores de diversos países consigam integrar as informações geradas para melhorar a produção do feijão-caupi (DIOUF, 2011).

Soares-Cavalcanti *et al.* (2010) analisaram em feijão-caupi os principais grupos de genes relacionados às vias de estresse abiótico (principalmente salinidade e seca) com ênfase no banco de dados do projeto "Genômica Funcional, Estrutural e Comparativa de Feijão-Caupi (*Vigna unguiculata*)" - NordEST. De forma geral, os dados disponíveis revelaram interessantes candidatos para o melhoramento do feijão-caupi e de outras leguminosas cultivadas, especialmente considerando a capacidade de adaptação a condições adversas desta espécie, que pode ser considerada como uma fonte ímpar de genes (e características) de resistência a estresses ambientais. Foram identificados genes estresse-induzidos que protegem diretamente contra os estresses ambientais e aqueles que regulam a expressão gênica e transdução de sinais na resposta ao estresse como Proteínas Quinase e Fatores de Transcrição. Wanderley-Nogueira et al. (2010) utilizaram os dados do banco NordEST para identificar proteínas de defesa de plantas conhecidas como proteínas relacionadas a patogênese (*Pathogenesis Related Protein*, PR – família PR2). Os autores identificaram cinco genes candidatos PR-2 e sugerem o desenvolvimento de marcadores moleculares com potencial para aplicação para o mapeamento genético e identificação de QTL.

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5. CAPÍTULO 1

Microsatellites, ISSR and DAF markers to study genetic relationships among *Vigna* species and its implication for cowpea breeding programs

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ABSTRACT

Genus *Vigna* includes several species of economic importance belongs to the family Fabaceae. Despite the importance of cowpea and related *Vigna* cultispecies, few data is available about the relationships among different Brazilian cowpea accessions to identify contrasting parents and increase the diversity of available genetic resistance to breeding program. The presented study aimed at accessing the genetic diversity across 23 cowpea (*V. unguiculata*) accessions as compared with six selected *Vigna* taxa and common bean as outgroup. For this purpose a three type molecular marker approach has been used, including 19 SSR (Simple Sequence Repeat), 21 ISSR (Inter Simple Sequence Repeat) and four DAF (DNA Amplification Fingerprinting. Besides clear separation from its relatives (*V. aconitifolia, V. angularis, V. mungo and V. radiata*), cowpea accessions could be clearly distinguished from native *V. unguiculata* subspecies (ssp. *cylindrical* and *sesquipedalis*) with high bootstrap value (100%). Furthermore, considerable levels of polymorphism could be detected within cowpea genotypes revealing contrasting parental candidates for biotic and abiotic resistance features with significant diversity.

Key words *Vigna*, DNA markers, Genetic relationships, germplasm, crop evolution.

1. Introduction

The legume genus *Vigna* was proposed by Savin in 1824 and comprises around 98 species classified into six subgenera distributed throughout Africa, America, Asia, Australia and the Pacific region (Onwueme and Sinha 1991; Tomoka et al. 2002; Thulin et al. 2004). Two of these subgenera have been classified by centers of origin: African *Vigna* species (*Vigna* group) and Asian *Vigna* species (*Ceratotropis* group). It contains several important cultivated species, including cowpea [*V. unguiculata* (L.) Walp.], mungbean [*V. radiata* (L.) Wilczek], adzuki bean [*Vigna angularis* (Willd.) Ohwi and Ohashi], blackgram [*V. mungo* (L.) Hepper], rice bean [*V. umbellata* (Thunb.) Ohwi and Ohashi] and moth bean [*V. aconitifolia* (Jacq.) Maréchal] (Pasquet 1996; Fery 2002). This genus proved to be interesting for various industrial applications all over the world especially in food industries and animal feed. There is currently an increased interest by scientific community for its ability to fix nitrogen symbiotically and its great adaptability to extreme environmental conditions (poor soils, dryness and high temperatures), which would normally limit the culture of other leguminous plants (Mbassi et al. 2011).

Cowpea is a multipurpose crop that ensures food security and improves high biological value for millions of people in Brazil and other parts of the developing world, because it contains substantial quantities of protein (about 25%), carbohydrate (about 64%), vitamins, minerals, lysine and tryptophan (Uarrota 2010; Hall 2012). The main use of cowpea as food regards dry grains, especially considering that this crop presents more resistance to terminal drought than either fresh peas or immature pods (Hall 2012).

In Brazil, the cowpea planting was initially promoted in the Northeast and North, where there are reports that this plant is well adapted to local climate conditions, being the principal subsistence crop in the semi-arid region. In recent years, cowpea has been grown and marketed in several areas of Central West (states of Goiás, Mato Grosso do Sul and Mato Grosso) with noticeably bright prospects in the Brazilian agribusiness. Low-technology cultivation techniques yield a national average of about 500 kg ha^{-1}. In the Northeast, where more than 90 % of the national production is growing, the average yield is only 317 kg ha^{-1} (Freire-Filho et al. 2005). The effect of field diseases on cowpea caused by viruses, root-knot nematodes and bruchid attack on stored grain has led to significant reduction in growth rate and productivity of cowpea, especially for the most productive cultivars (Timko and Singh 2008).

Molecular genetic tools and genomic resources have been developed for cowpea aiming to enhance breeding success for the improvement of cowpea varieties for the United States, India, Brazil, and numerous countries in Africa and Asia (Pottorff et al. 2012). Brazilian cowpea

breeding program needs also to develop robust sets of genetic markers for accurately assessing genetic diversity in the course of selection programs, germplasm evaluations and planning of crosses in order to generate new cultivars with different desired attributes and resistance to major biotic and abiotic stresses, in this last case mainly drought and salinity. In other countries, especially India and Africa, recent studies have assessed the genetic diversity of cowpea using different molecular tools, such as RAPD (Randomly Amplified Polymorphic DNA) markers (Ba et al. 2004; Zannou et al. 2008; Malviya et al. 2012; Prasanthi et al. 2012), AFLP (Amplified Fragment Length Polymorphism) (Fang et al. 2007), SSR (Li et al. 2001; Diouf et al. 2005; Uma et al. 2009; Asare et al. 2010; Gupta and Gopalakrishna, 2010) and ITS (Internal Transcribed Spacer) (Vijaykumar et al. 2012). Most studies revealed low genetic diversity among accessions from the same country. In Brazil, three evaluations with random markers (Xavier et al. 2005; Simon et al. 2007; Spiaggia et al. 2009) were noteworthy to determine the genetic variability of the available accessions in order to introduce these plants into breeding programs. Unfortunately, only a limited number of molecular markers have been developed and used for breeding program including Brazilian cowpea accessions and many important accessions for the breeding program were not included.

The purpose of this study was to investigate genetic relationships among cowpea accessions and *Vigna* species, with emphasis on Brazilian and African contrasting cowpea cultivars in order to establish the existing diversity and the utility of different marker systems. For this mean, 23 selected cowpea genotypes were compared to six selected *Vigna* taxa, in order to contribute to germplasm bank management and conservation programs. Furthermore, this approach intended to identify contrasting parental lines to generate segregating populations for features including resistance/tolerance to both biotic and abiotic stresses.

2. Material and methods

2.1. Plant material and Genomic DNA extraction

In this study 29 genotypes of seven different *Vigna* species were analyzed. These comprised 23 accessions of the African subgenus *Vigna* (23 *V. unguiculata* and two subspecies *cylindrica* and *sesquipedalis*) and also a single accession of each of the four species the Asian subgenus *Ceratotropis* (*V. aconitifolia*, *V. angularis*, *V. mungo* and *V. radiata*) obtained from EMBRAPA CPAMN (Centro de Pesquisa Agropecuária do Meio-Norte, Terezina, Piauí state,

Brazil), IPA (Instituto Agronômico de Pernambuco, Recife, Pernambuco state, Brazil), UFC (Univerdade Federal do Ceará, Ceará State, Brazil) and IPK (Institut für Pflanzengenetik und Kulturpflanzenforschung Gatersleben, Germany). Additionally, two cultivars of *Phaseolus vulgaris* L. ssp. *vulgaris* ('Neckarkönigin' and 'Delinel') were employed as outgroup for the phylogenetic analysis (Table 1).

Prior to sowing, the seeds were surface-sterilized with 4% sodium hypochlorite, and 4 plants per pod were cultivated in 5 kg of a mixture of two parts soil and one part manure. Young leaf tissues (4 g) from five-day-old seedlings were ground in liquid nitrogen by using pre-chilled mortar and pestle, and the DNA was isolated using a maxi-prep cetyl trimethyl ammonium bromide (CTAB) protocol (Weising et al. 1995). Contaminating polysaccharides were selectively precipitated (Michaels et al. 1994). The pellet was dissolved in TE buffer (pH 8.0) and subjected to RNAase treatment for purification. DNA concentrations were determined electrophoretically in 1.2 % agarose gel using known amounts of phage λ DNA (MBI, Fermentas, Hanover, MD, USA) as reference.

2.2. SSR analysis

Twenty primer pairs were used to assess the transferability and the level of polymorphism among cowpea cultivars and related species. Of these, nine SSR primer pairs were developed by Li et al. (2001) with microsatellite-enriched libraries of cowpea (VM primers), five were developed by Wang et al. (2004) using (AG)n-SSR-enriched library in *V. angularis* (CEDG primers) and five were designed by Gwag et al. (2006) with microsatellite-enriched libraries of *V. radiata* (GB-SSR). Reactions were carried out at a final volume of 20 μL, including 2.5 μM $MgCl_2$, 1x PCR buffer, 200 μM dNTP-mix (Fermentas), 0.5 U of *Taq* DNA polymerase (Fermentas) and 30 ng of genomic DNA.

Reactions using primers developed by Li et al. (2001) and Gwag et al. (2006) included 10 pmol of each forward and reverse primer, with an initial denaturation at 94°C for 5 min, followed by 38 cycles of denaturing at 94ºC for 1 min; annealing at 54 to 60°C (depending on the primer) for 30 s and extension at 72ºC for 1 min, with a final extension at 72ºC for 5 min. For the reactions using primers designed by Wang et al. (2004) 5 pmol of each forward and reverse primers was used. The temperature cycling profile involved an initial denaturation step of 2 min at 94ºC. This was followed by 30 cycles at 94ºC for 15 s, an annealing phase of between 68ºC for 15 s, and a final extension at 68ºC for 5 min.

The generated SSR fragments were separated in 5% non-denaturing polyacrylamide gels and were visualized after staining with silver nitrate according to Creste et al. (2001). A 100 pb DNA ladder (New England Biolabs or Fermentas) was used as a molecular marker standard.

2.3.DAF analysis

Four DAF (DNA Amplification Fingerprinting) primers. The reactions were performed according to Simon *et al*. (2007), using 1 ng of genomic DNA, 1x PCR buffer, 2.5 mM $MgCl_2$, 10 mM dNTP-mix, 50 pmol primer and 0.5 U *Taq* DNA polymerase. The final volume was adjusted to 15 μL with Milli-Q H_2O. The PCR reaction was carried out in an Eppendorf Mastercycler gradient PCR machine (Hamburg, Germany) as follows: an initial denaturation for 2 min at 95ºC followed by 40 cycles at 95ºC for 15 s, 35ºC for 1 min, 72ºC for 2 min with a final elongation at the same temperature for 2 min. The amplified products were resolved on ethidium bromide-stained 1.8% agarose gel, using 0.5x TBE buffer, at 70 V for 4 hours. Gel images were acquired through UV light using. The molecular size of the amplicons was determined with reference to the 100 bp DNA ladder (Fermentas).

2.4 ISSR analysis

A total of twenty-two primers were applied using ISSR (Inter Simple Sequence Repeat, University of British Columbia, Canada) analysis that included 19 anchored primers (dinucleotide repeats) three non-anchored primers (two with tri-nucleotide and one with penta-nucleotide repeats), primers ordered from MWG-Biotech. The ISSR reactions were performed following the protocol described by Costa et al. (2011). In 20 μL reactions the volume contained 15 ng of genomic DNA, 1x buffer, 2.5 mM $MgCl_2$, 200 μM of each dNTP, 50 pmol primer and 0.5 U *Taq* DNA polymerase. Amplifications were performed in an Eppendorf Mastercycler gradient PCR machine programmed for an initial denaturation at 94ºC for 4 min, 30 cycles at 94ºC for 30 s, 50.4 to 60.5°C depending on the primer for 1 min, 72ºC for 2 min, and a final extension at 72ºC for 7 min. Amplified fragments were separated in agarose (1.8%) gel electrophoresis stained with ethidium bromide. The bands were visualized and acquired under UV light using a 100 bp ladder (Fermentas) as size reference.

2.5. Genetic diversity analysis

As applied by Tatikonda et al. (2009), the discriminatory power of ISSR and DAF primers was determined by using three parameters: (1) polymorphism information content (PIC), (2) marker index (MI), and (3) resolving power (RP). PIC values were obtained according to Roldán-Ruiz et al. (2000): $PIC_i = 2\ f_i\ (1 - f_i)$, where PIC_i regards the polymorphism information content of marker i, f_i the frequency of present of marker fragments that were and $1 - f_i$ the frequency of absent marker fragments. Subsequently, the average PIC was obtained for each primer combination/single primer. MI values were obtained as described by Varshney et al. (2007): $MI = PIC \times n \times n_p/(n_p + n_m)$, where n is the average number of fragments per primer, n_p the number of polymorphic fragments and n_m the number of monomorphic fragments. RP was calculated according to Prevost and Wilkinson (1999): $RP = \Sigma\ I_b$, where I_b represents fragment informativeness. The I_b can be represented into a 0–1 scale by the following formula: $I_b = 1 - (2 \times |0.5 - f_i|)$. Additionally, for the SSR markers, the discriminatory power of the primer pairs was determined by calculating PIC values, according to Botstein et al. (1980) and to Nei's unbiased gene diversity (Nei 1987) – the expected heterozygosity.

Genetic distances among accessions were estimated according to the Dice's coefficient (Perrier et al. 2003) with DARwin 5.0 (Perrier and Jacquemoud-Collet 2006). Then, dissimilarity matrixes with data from the three marker systems were obtained and used to generate four phenograms [(1) ISSR markers, (2) DAF markers, (3) ISSR+DAF and (4) SSR markers] with the neighbor-joining algorithm (bootstrap with 1,000 replications).

3. Results and Discussion

All the primers used in the study generated considerable polymorphisms among *Vigna* species. The screening with 22 ISSR primers generated 729 scorable band classes, among which 644 comprised polymorphic markers for *Vigna*. As an example, the pattern obtained for each cultivar with primer UBC888 is shown in Fig. 1. The number of polymorphic bands at the intraspecific level in cowpea cultivars produced by a single primer varied from one (UBC 810) to eighteen (UBC 888), with an average frequency of 5.8 polymorphic bands per primer. Five (UBC 826, 840, 846, 866, 885) out of 22 ISSR primers produced monomorphic profiles in cowpea (Table 2).

In a general way, primers containing AC or AG repeats provided higher polymorphism. Ghalmi et al. (2010) also observed high percentage of polymorphism in cowpea landraces with the dinucleotide repeats GA (74.7%), AG (61.11%) and AC (57.69%). These results agree to the earlier studies in *Vigna* where GA and CA repeats have been reported to generate high polymorphism rates among *Vigna* genotypes (Ajibade et al. 2000). In blackgram [*Vigna mungo* (L.) Hepper] poly 'GA' and poly 'AG' primers also produce higher number of amplicons and polymorphic bands (Souframanien and Gopalkrishna 2004).

The primer UBC 835 revealed the highest polymorphic information content (PIC value, 0.21), marker index (MI value, 2.36), and resolving power (Rp, 3.21) among 23 cowpea genotypes. The primer UBC 861 presented the lowest value of average PIC (0.02), MI (0.06), and RP (0.34). The average PIC value of 0.075 across all scored ISSR bands were lower than other results obtained for *Vigna* species.

Tantasawat et al. (2010a) used 16 polymorphic ISSR markers to distinguish 28 yardlong bean (*Vigna unguiculata* spp. *sesquipedalis*) and dwarf yardlong bean accessions. They obtained PIC values from 0.137 to 0.276 with an average of 0.197 among genotypes. Tantasawat et al. (2010b) studied the genetic diversity of blackgram and mungbean with 18 ISSR primers; they found PIC values from 0.23 to 0.37, with an average of 0.31 across 17 mungbean and 5 blackgram genotypes. In comparison to our results, the PIC values detected by Tansawat et al. (2010a; 2010b) was higher probably due to the use of widely distributed accessions from geographically worldwide origin, besides the use of a number of selected highly informative primers, which have been used successfully in blackgram and *Vigna* species (Ajibade et al. 2000; Souframanien and Gopalakrishna 2004). On the other hand, our results have shown higher frequency of polymorphic bands in *Vigna* species than previous reports also bearing significant polymorphism within cowpea accessions (ranging from 16.7 to 90%). Such evidences suggest that informative ISSRs can be applied to genetic breeding and mapping. The number of polymorphic bands per primer was higher (5.8) than that obtained by Souframanien and Gopalakrishna (2004), who found 3.4 polymorphic bands per primer, being similar to those found by Niharika et al. (2010) and Priyanka et al. (2012) that observed 6.25 and 6.5 polymorphic bands per primer, respectively. The low PIC values observed in the polymorphic with higher frequency is to be expected because these bands were more prevalent among accessions, tending towards monomorphism. On the other hand, for loci where bands are amplified with lower frequency, the absence of bands would be more prevalent among accessions, also tending towards monomorphism. Nevertheless, these bands are important for understanding the relationships and similarities between accessions (Gravitol et al. 2010).

The high polymorphism number, revealed by the ISSR technique, was not unexpected, since the technique amplifies microsatellite areas that are potentially variable, as observed previously in common bean (Marotti et al. 2007), in the genus *Cicer* and in cultivated chickpea (Rao et al. 2007), in lentil (Sonnante and Pignone 2007), in field pea (Kapila et al. 2012) and also in blackgram (Kanimozhi et al. 2009; Karuppanapandian et al. 2010). The potential supply of ISSR markers depends on the variety and frequency of microsatellites, which changes with species and the SSR motifs that are targeted (Depeiges et al. 1995).

In the DAF analyses, four primers used generated reproducible, informative and easily scrabble DAF profiles, producing multiple band profiles (Figure 1) with 161 bands and 146 polymorphisms for *Vigna*. The number of polymorphic bands generated by a single primer varied from 28 to 47 among *Vigna* species, and from 6 to 21 (with an average of 16.5) at the intraspecific level among cowpea accessions. In particular, primers OP-G06 and OP-K14 produced the largest number of bands (52 and 44, respectively), while primer OP-G06 detected the highest level of interspecific and intraspecific polymorphism (Table 3). Our approach was definitely more efficient for the detection of polymorphisms than observed by Xavier et al. (2005), where the authors used 20 primers RAPD to evaluate 45 cowpea genotypes. From these, eight polymorphic primers were identified, comprising a total of 48 informative bands. The success of our study in identifying polymorphism is due to the use of a number of randomly selected prescreened highly informative primers used by Simon et al. (2007). These results are in agreement with the report of Malviya et al. (2012), who observed that significant polymorphism in cowpea genotypes could be produced by use of pre-screened, highly-informative RAPD primers of another grain legume, pigeon pea [*Cajanus cajan* (L.) Millsp.].

The respective values for overall genetic variability for PIC, MI and Rp across all 23 cowpea genotypes are given in Table 3. Highest PIC value (0.16) was observed for the primer OPG06 and lowest value (0.10) was recorded for the primer OPK14. The MI values ranged from 1.75 (OPK14) to 2.29 (OPB07). The highest RP value (3.47) was scored with the primer OPG06 and the lowest (2.17) for the primer OPB07. Nevertheless, on the basis of higher PIC average values (DAF=0.126; ISSR=0.075), MI average values (DAF =1.99; ISSR= 0.526) and Rp average values (DAF= 2.73; ISSR= 1.23), the DAF markers were marginally more informative than ISSR in the assessment of genetic diversity in cowpea cultivated. The similar results are reported for *Vigna umbellata* (Muthusamy et al. 2008) with RAPD and ISSR markers. This is opposite to the results as obtained for several other plant species like wheat (Nagaoka and Ogihara 1997) and *Vigna* (Ajibade et al. 2000). The two-marker techniques target different portions of the genome, what may explain the contrasting resolution of DAF and ISSR markers

(Gupta et al. 2010). Polymorphic bands of a given DAF primers may bind to many parts of the genome. So, each primer covers a larger proportion of the genome, including coding and non-coding regions and may give information on the polymorphism of several chromosome regions, as observed in chickpea by Benko-Iseppon et al. (2003). ISSR primers also provide a large quantity of polymorphic information, but this detailed information is only originated from a single hypervariable section of the genome (Meszaros et al. 2007). The number of loci and their coverage of the overall genome are important to obtain reliable estimatives of genetic relationships among crops (Gupta et al. 2008). Therefore, both ISSR and DAF markers were extremely useful to select cowpea parental candidates to be crossed for generating appropriate populations for mapping and breeding purposes.

The positive correlation between the PIC and MI values ($r = 0.9068$; $p < 0.0001$), and the RP and MI values ($r = 0.8747$; $p < 0.0001$) observed in our study, indicates the usefulness of MI parameters to compare the information content of polymorphic ISSR and the use of RP to select the most informative ISSR marker to distinguish among different accessions. The markers with high RP values were more informative, being able to distinguish a higher number of cowpea genotypes. Considering DAF markers, the absence of positive correlation between PIC, MI and RP might be attributed to the band distribution and total number of alleles within the sampled genotypes.

Using the SSR methodology with 19 primer pairs developed from microsatellite-enriched libraries of cowpea, adzuki bean and mungbean, a total of 175 polymorphisms were detected , 89 at intraspecific level, among cowpea genotypes and 156 at interspecific level. Cowpea-derived SSR primers amplified microsatellite sequences from the majority of the *Vigna* accessions assessed in the present study. Four out of the nine cowpea SSR primer-pairs (VM10, VM31, VM36, VM37) were 100% successfully transferable across the *Vigna* species. Furthermore, four cowpea SSR primer-pairs produced >80% positive amplifications and one cowpea primer-pair was 40% successful. For Adzuki bean SSR primers, the highest level of successful amplification was 100 and 90% generated by three (CEDG043, CEDG026, CEDG015) and two (CEDG008 and CEDG024) primer pairs, respectively. As expected, the transferability rate of the markers was high, indicating that the flanking regions of these SSR loci were sufficiently conserved, and could be used for comparative analyses of genetic diversity and comparative linkage mapping in different *Vigna* species.

The individual mean percentage of successful transferability of cowpea SSR primers across adzuki bean, mungbean, moth bean species was 78% and 67% for blackgram. For the adzuki bean SSR primers, it was 100% for cowpea and 80% for mungbean, moth bean and

blackgram. The higher transferability of cowpea SSR primers to the genotypes studied here was not surprising, especially in cowpea accession and for the subspecies of *V. unguiculata*, indicating that these sequences are conserved at intraspecific level. The remarkable level of transferability supports previous findings in *Vigna* species. Wang et al. (2009) reported transferability of adzuki bean primers in mungbean and found that 70% of them generated clear and repeatable bands in mungbean. Dikshit et al. (2012) evaluated the transferability of adzuki bean SSR to mungbean and related *Vigna*. The transferability percentage across the genotypes ranged from 60.97 to 92.6% with 87.8% in *V. radiata* and *V. mungo*, 62.2% in *V. unguiculata*, 91.8% in *V. umbellata*, 78% in *V. mungo* var. *sylvestris* and 80% in *V. trilobata*, respectively.

The PIC value of these 19 SSR markers was computed to be 0.828 (VM10), while the minimum was 0.347 (VM27), with a mean of 0.468. The PIC values of SSR markers can be compared to the results reported by Gupta and Gopalakrishna (2010) with PIC ranged from 0.10 to 0.83. Asare et al. (2010) reported a PIC ranging from 0.07 to 0.66 with a mean of 0.38 among Ghanaian cowpea accessions. Gene diversity (H_E) was 0.507 on average. VM10 marker exhibited the highest gene diversity (H_E) with 0.84, while the least was CEDG008 detecting 0.383 (H_E). Low levels of heterozygosity (H_O) were observed, varying from 0.10 (GBssr-MB14) to 1.00 (VM39), with a mean of 0.149, while some of the markers detected no heterozygozity (Table 4). Also, similar to the findings of Tangphatsornruang et al. (2009), the observed heterozygosity within mungbean accessions was low for all genotypes examined, which is most likely due to the inbreeding nature of cowpea and mungbean.

The dissimilarity matrixes based on combined data generated from dominant (DAF and ISSR) and co-dominant markers (SSR) data sets is graphically represented as different phenograms using the neighbor-joining method, as shown in Figs. 3 and 4. For both marker types, two distinct phylogenetic groups were identified: a small cluster into which the *Phaseolus* grouped and a larger cluster that contained the all *Vigna* accessions. In the *Vigna* group two distinct branches formed within the larger group, one for the Asian *Vigna* species subgenus *Ceratotropis*, with *V. aconitifolia* in a basal position, from which a cluster emerged including *V. mungo, V. radiata, V. angularis* and a branch including all African accessions of *V. unguiculata*, whereas the wild subspecies *V. unguiculata* ssp. *cylindrica* and *V. unguiculata* ssp. *sesquipedalis* appeared separately as basal branch.

Grouping structure nearly reflected the present-day taxonomic relationships reflected by previous works using RAPD, RFLP, ISSR and SSR analysis (Fatokun et al. 1993; Kaga et al. 1996; Ajidabe et al 2000; Phansak et al 2005; Simon et al. 2007), where the Asian and African

Vigna groups were clearly differentiated. Within the Asian *Vigna* group, *V. radiata* and *V. mungo* clustered closely to *V. aconitifolia*.

In our evaluation the combined analysis of *Vigna* species was based on a single accession of each species and 23 for cowpea. Further analysis using several accessions per species may produce a more accurate identification of the genetic diversity within and among these species. Ajibade et al. (2000) used nineteen ISSR primers to study the genetic relationships among 18 *Vigna* species. The ISSR polymorphisms produced by 15 of these primers were very effective for distinguishing taxa at the species level or below. The study revealed that closely related species within each subgenus clustered together (e.g., *V. umbellata* and *V. angularis* (subgenus *Ceratotropis*), *V. adenantha* and *V. caracalla* (subgenus *Sigmoidotropis*), and *V. luteola* and *V. ambacensis* (subgenus *Vigna*). Cultivated cowpea accessions grouped closely to the wild subspecies of *V. unguiculata*, and the entire species was separated from its most closely allied species *V. triphylla* and *V. reticulate.* Therefore, the authors note that their conclusions regarding subgeneric classifications should be taken with caution, due to the low number of analyzed genotypes.

Phansak et al. (2005) applied SSR markers to determine the level of genetic variation among yardlong bean accessions and to analyze the genetic relatedness between yardlong bean to six other cultivated *Vigna* species, including mungbean, blackgram, rice bean, adzuki bean, moth bean and cowpea. Group A represented the African *Vigna* species yardlong bean and cowpea, while groups B (mungbean, blackgram and moth bean) and C (rice bean and adzuki bean) represented the Asian *Vigna* species.

Improvement in the current crop status requires resistance to biotic and abiotic stresses, which are fundamental causes for unstable production. The cultivated taxon lacks adequate sources of resistance to these stresses. The success of the large-scale adoption and commercialization of cowpea in the Northeast region of Brazil, is crucially dependent on resistance to viruses (caused by *Cowpea severe mosaic virus*, CPSMV and *Cowpea aphid borne mosaic virus*, CABMV). Worldwide, root rust caused by a nematode (*Meloidogyne* spp.) is a serious disease and *Callosobruchus* limited damage during storage of cowpea seeds. Wild relatives of chickpea have been shown to be promising sources for genes for resistance to the major biotic and abiotic stresses in chickpea (Singh et al., 1994).

The phenogram performed on combining data of both ISSR and DAF markers demonstrates that the cowpea accessions could be classified into two major branches with a bootstrap value of 92%. One contained two subgroups represented the African cowpea accessions (TVu 779, TVu 382, IT86D-716-1 and IT85F-2687) identified as source of resistance

for CABMV and CPSMV viruses (Lima et al. 1986, Rocha et al. 1996, Lima et al. 1998), except for IT85F-2687 that is sensitive to CPSMV. The second major group comprised 19 Brazilian accessions with diversity in response to biotic and abiotic stresses into three subgroups. In the first subgroup, a single accession (TE96-282-22G) formed a distinct and separate cluster. Subgroup 2 contained three Brazilian accessions (CE 315, BR9 Longá and IT81D-1053). Subgroup 4 contained fifteen accessions; most of them are Brazilian accessions except (Vita 3 and Vita 5). BR14-mulato (resistant CPSMV) has been crossed with IT85F-2687 to develop recombinant inbred lines for identifying DNA markers linked to virus resistance and to transfer the character into ye background to develop multiple biotic-resistant genotypes. Further studies are envisaged to quantify the genetic gain in populations derived from genotypes with distinct DNA profiles. This is also the case of accessions IT81D-1053 and CE31 are contrasting for weevil (*Callosobruchus maculatus*). *Callosobruchus* spp., causes serious damage to several leguminous crops including cultivated *Vigna* species, such as mugbean, azuki bean, and cowpea during storage.

Differential responses to abiotic stress were observed among clusters. The association of accessions with salinity tolerance (Pitiúba, Epace 10 and IPA 201) in the same cluster, was supported by high bootstrap scores (77%), indicating that these accessions are also interesting for breeding of this feature (Gomes-Filho et al. 2002; Pimentel et al. 2002). Considering salinity sensitive accessions, two groups had been formed: the first containing three accessions (IPA 204, IPA 205 and IPA 206) and the second with two accessions (Canapu Amarelo and Pérola). Other authors have shown that the genetic variations identified by microsatellite markers are useful in evaluating upland accessions for drought-tolerance related morphology (Zeng et al. 2004; Manavalan et al. 2009; Vanniarajan et al. 2012).

When phenograms were compared, the clustering of genotypes within groups was not similar when dominant (DAF + ISSR) and co-dominat (SSR) markers were used. The differences found among the phenograms generated by dominant and co-dominant could be partially explained by the different number of polymorphisms generated, what reinforces the importance of loci number and overall genome coverage to obtain a reliable estimation of genetic relationships among cowpea genotypes. However, the information from genetic classification obtained from dominant and co-dominant markers type would be useful for choosing parental candidates to develop crosses for cowpea breeding and to classify cowpea accessions in germplasm management.

Previous generation of genetic maps in cowpea were sometimes difficult, with most authors arguing that cowpea could bear a genetic narrow basis (Menancio-Hautea 1993;

Menéndez et al. 1997). Despite of that, our results showed that DAF, ISSR and SSR techniques were efficient to generate informative molecular markers and that diversity is larger than that observed in other legumes. This brings promissory perspectives, providing important data for selection of contrasting parental lines for the construction of a map with higher marker density. Accessions with the most distinct DNA profiles are likely to contain the greatest number of novel alleles, with higher perspectives to uncover the largest number of unique and potentially agronomic useful alleles (Souframanien and Gopalakrishna 2004).

Considering the application of the present data to cowpea breeding, our results reveal that the Brazilian germplasm comprises relatively high levels of genetic diversity to design crosses for mapping purposes employing linkage analysis of QTLs (Quantitative Trait Loci) and also fine-mapping to identify genes governing important traits with aid of DNA markers.

The informative primers identified in our studies will be useful in genetic analysis of *Vigna* accessions in germplasm holdings. The putative species-specific bands can be used as probes to ascertain whether they are in low or high copy numbers in the *Vigna* genome, and such specific bands may be useful for genotype characterization and grouping of germplasm accessions. Further, putative species-specific DAF and ISSR markers could be converted to sequence characterized amplification regions (SCARs) after sequencing and primer designing to develop robust species specific markers. The study also provides a basis for *Vigna* breeders to make informed choices on selection of parental material based on genetic diversity to help overcome some of the problems usually associated with a tree crop improvement program.

Like in the case of others crops, in the actual conjecture, cowpea breeding in Brazil aims to obtain varieties with resistance against different pathogens, as well as against abiotic stresses incorporated in cultivars with important agronomic characters. The establishment of genetic maps for such interesting characters, as well as the establishment of associated markers constitute actions that will facilitate the research works, placing cowpea in the future among the improved crops benefiting from molecular breeding and biotechnology.

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Table 1. Accession numbers and sources of *Vigna* and *Phaseolus* genotypes analyzed in the present study. l. EMBRAPA = Empresa Brasileira de Pesquisa Agropecuária, Centro de Pesquisas do Agropecuária do Meio-Norte (CPAMN), Piauí, PE, Brazil; Instituto Agronômico de Pernambuco (IPA), Recife, PE, Brazil; Univerdade Federal do Ceará (UFC), Ceará, CE; Institut für Pflanzengenetik und Kulturpflanzenforschung Gatersleben (IPK), Germany. Accessions with no germplasm designations were acquired on local markets. Important agronomic traits considered only for cultivated cowpea accessions.

Taxon	Accession Nr.	Germplasm bank	Important Agronomic Traits
V. unguiculata (L.) Walp. ssp. *unguiculata*	Pitiúba	UFC	Tolerance Abiotic Stress (Salt/Drought)
V. unguiculata (L.) Walp. ssp. *unguiculata*	Epace 10	IPA	Tolerance Abiotic Stress (Salt/Drought)
V. unguiculata (L.) Walp. ssp. *unguiculata*	IPA 201	IPA	Susceptible CPSMV
V. unguiculata (L.) Walp. ssp. *unguiculata*	Vita 3	EMBRAPA	Tolerance Abiotic Stress (Salt)
V. unguiculata (L.) Walp. ssp. *unguiculata*	Sempre Verde	EMBRAPA	Susceptible CPSMV
V. unguiculata (L.) Walp. ssp. *unguiculata*	Vita 5	EMBRAPA	Tolerance Abiotic Stress (Salt)
V. unguiculata (L.) Walp. ssp. *unguiculata*	BR17 Gurguéia	EMBRAPA	Resistant CPSMV
V. unguiculata (L.) Walp. ssp. *unguiculata*	Canapu Amarelo	EMBRAPA	Sensitive Abiotic Stress (Salt/Drought)
V. unguiculata (L.) Walp. ssp. *unguiculata*	Pérola	EMBRAPA	Sensitive Abiotic Stress (Salt/Drought)
V. unguiculata (L.) Walp. ssp. *unguiculata*	CNC0434	IPA	Resistant CPSMV
V. unguiculata (L.) Walp. ssp. *unguiculata*	BR14 Mulato	EMBRAPA	Resistant CPSMV, Sensitive CABMV
V. unguiculata (L.) Walp. ssp. u*nguiculata*	IPA 204	IPA	Sensitive Abiotic Stress (Salt) Susceptible CPSMV
V. unguiculata (L.) Walp. ssp. *unguiculata*	IPA 205	IPA	Sensitive Abiotic Stress (Salt) Susceptible CPSMV
V. unguiculata (L.) Walp. ssp. *unguiculata*	IPA 206	IPA	Sensitive Abiotic Stress (Salt)
V. unguiculata (L.) Walp. ssp. *unguiculata*	TE 96.282.22G	EMBRAPA	Resistant CABMV
V. unguiculata (L.) Walp. ssp. *unguiculata*	CE 31	UFC	Susceptible Nematoid *(M.incognita)*
V. unguiculata (L.) Walp. ssp. *unguiculata*	BR9 Longá	UFC	Susceptible Weevie (*C. malucatus)*
V. unguiculata (L.) Walp. ssp. *unguiculata*	CE315	UFC	Resistant Nematoid *(M.incognita)* Susceptible CPSMV
V. unguiculata (L.) Walp. ssp. *unguiculata*	IT81D 1053	EMBRAPA	Resistant Weevil (*C. malucatus)*
V. unguiculata (L.) Walp. ssp. *unguiculata*	IT85F 2687	EMBRAPA	Sensitive CPSMV Resistant CABMV
V. unguiculata (L.) Walp. ssp. *unguiculata*	IT86D 716 1	EMBRAPA	Resistant CABMV
V. unguiculata (L.) Walp. ssp. *unguiculata*	TVU 382	EMBRAPA	Resistant CPSMV
V. unguiculata (L.) Walp. ssp. *unguiculata*	TVU 379	EMBRAPA	Resistant CPSMV
V. ung. (L.) Walp. ssp. *cylindrica* (L.) Verdc.	VIG 79/82	IPK	-
V. ung. (L.) Walp. ssp. *sesquipedalis* (L.) Verdc.	VIG 28/76	IPK	-
V. angularis (Willd.) Ohwi et Ohashi	Azikihnen	IPK	-
V. radiata (L.) Wilcz	Mungbohnen	IPK	-
V. mungo (L.) Hepper	PHA 81 48/85	IPK	-
V. aconitifolia (Jacq.) Maréchal	PHA 8150/80	IPK	-
Phaseolus vulgaris	Neckar	IPK	-
Phaseolus vulgaris	Delinel	IPK	-

Table 2. List of ISSR primers used and their nucleotide sequence, including the total number of amplified and polymorphic bands and PIC values at intra and interspecific level.

Primers	Repeats	Total *A*	Intraspecific Level Between Cowpea accessions						Interspecific Level Between Genus *Vigna*			
			A	*P*	f_p (%)	*PIC*	*MI*	*RP*	*A*	*P*	f_p (%)	*PIC*
ISSR807	$(AG)_8T$	41	13	6	46.1	0.146	0.821	2.435	34	34	100	0.135
ISSR810	$(GA)_8T$	21	6	1	16.7	0.076	0.154	0.696	18	18	100	0.133
ISSR811	$(GA)_8C$	41	21	15	71.4	0.077	0.670	1.739	39	39	100	0.107
ISSR825	$(AC)_8T$	45	18	16	88.9	0.099	1.069	1.913	40	40	100	0.097
ISSR826	$(AC)_8C$	33	6	0	0	-	-	-	30	30	100	0.101
ISSR828	$(TG)_8A$	27	10	4	40.0	0.150	0.730	2.174	22	22	100	0.166
ISSR834	$(AG)_8YT$	39	13	7	53.8	0.116	0.759	1.913	35	35	100	0.126
ISSR835	$(AG)_8YC$	22	10	9	90.0	0.216	2.363	3.217	16	16	100	0.160
ISSR840	$(GA)_8YT$	28	9	0	0	-	-	-	25	25	100	0.126
ISSR841	$(GA)_8YC$	39	14	9	64.3	0.177	1.387	3.652	33	33	100	0.152
ISSR846	$(CA)_8RT$	29	11	0	0	-	-	-	29	29	100	0.136
ISSR847	$(CA)_8RC$	24	11	5	45.5	0.083	0.461	1.043	20	20	100	0.139
ISSR848	$(CA)_8RG$	25	11	6	54.5	0.064	0.425	0.783	21	21	100	0.124
ISSR857	$(AC)_8YG$	32	12	3	25.0	0.068	0.207	1.304	30	30	100	0.144

Continues in the next page.

Continuation of table 2:

Primers	Repeats	Total *A*	Intraspecific Level Between Cowpea accessions						Interspecific Level Between Genus *Vigna*			
			A	*P*	f_p (%)	*PIC*	*MI*	*RP*	*A*	*P*	f_p (%)	*PIC*
ISSR861	$(ACC)_6$	38	11	2	18.2	0.028	0.062	0.348	34	34	100	0.117
ISSR866	$(CTC)_6$	29	8	0	0	-	-	-	26	26	100	0.124
ISSR880	$(GGAGA)_3$	34	14	10	71.4	0.075	0.653	1.130	31	31	100	0.096
ISSR884	$HBH(AG)_7$	28	13	7	53.8	0.056	0.370	0.783	24	24	100	0.124
ISSR885	$BHB(GA)_7$	32	10	0	0	-	-	-	28	28	100	0.130
ISSR887	$DVD(TC)_7$	35	12	5	41.7	0.069	0.352	1.304	30	30	100	0.125
ISSR888	$BDB(CA)_7$	57	25	18	72.0	0.094	0.828	2.957	52	52	100	0.120
ISSRK1	$HVH(CA)_7$	30	10	4	40.0	0.055	0.269	0.609	27	27	100	0.122
Total	- -	729	268	127	47.39	-	-	-	644	644	100	-
Mean	- -	33.14	12.18	5.77	40.61	0.075	0.526	1.273	29.27	29.27	100	0.127

Note: ISSR primers were obtained from the University of British Colombia. R = (A,G); Y = (C,T); B = (C,G,T) (i.e. not A); D = (A,G,T) (i.e. not C); H = (A,C,T) (i.e. not G), V = (A,C,G) (i.e. not T). *A*, Amplified bands; *P*, Polymorphic bands; f_p, Polymorphic frequency; *PIC*, Polymorphism Information Content; *MI*, marker index; *RP*, resolving power.

Table 3. List of DAF primers used and their nucleotide sequence, including the total number of amplified and polymorphic bands and PIC values at intra and interspecific level.

Primers	Sequence (5'→3')	Total *A*	Intraspecific Level (between Cowpea accessions)						Interspecific Level (among *Vigna* species)			
			A	*P*	f_p (%)	*PIC*	*MI*	*RP*	*A*	*P*	f_p (%)	*PIC*
OP-B07	GGT GAC GCA G	33	8	6	75	0.165	2.295	2.174	30	30	100	0.238
OP-G06	GTG CCT AAC C	52	23	21	91.3	0.124	2.094	3.478	47	47	100	0.067
OP-K14	CCC GCT ACA G	44	22	20	90.9	0.104	1.757	2.522	41	41	100	0.067
OP-R26010	GAC CGA CAC G	32	20	18	90.5	0,109	1.826	2.783	28	28	100	0.067
Total		161	73	65	89.04	-	-	-	146	146	100	-
Mean		40.25	18.25	16.25	86.92	0.126	1.993	2.739	36.5	36.5	100	0.109

A, Amplified bands; *P*, Polymorphic bands; f_p, Polymorphic frequency; *PIC*, Polymorphism Information Content; *MI*, marker index; *RP*, resolving power.

Table 4. Original primers used in SSR methodology, with their sequences, including the repeat motif, total number of alleles, number of expected and observed heterozygosity and PIC values at the intraspecific level between Cowpea accessions.

Primers	Sequence (5'→3')	Repeat motif	H_E	*Ho*	*PIC*	*NA*
VM 05	L - AGCGACGGCAACAACGAT R - TTCCCTGCAACAAAAATACA	$(AG)_{32}$	0.767	0	0.710	5
VM 10	L - TCCCACTCACTAAAATAACCAACC R - GGATGCTGGCGGCGGAAGG	$(AC)_3(CT)_{10}(AC)_3$	0.865	0.957	0.828	10
VM 27	L - GTCCAAAGCAAATGAGTCAA R - TGAATGACAATGAGGGTGC	$(AAT)_5...(TC)_{14}.(AC)_3$	0.405	0	0.347	3
VM 31	L - CGCTCTTCGTTGATGGTTATG R - GTGTTCTAGAGGGTGTGATGGTA	$(CT)_{16}$	0.769	0	0.728	7
VM 32	L - GAAAAAGGGAGGAACAAGCACAAC R - AGCGAAAACACGGAACTGAAATC	$(AG)_{10}$	0.778	0	0.724	6
VM 36	L - ACTTTCTGTTTTACTCGACAACTC R - GTCGCTGGGGGTGGCTTATT	$(CT)_{13}$	0.828	0.043	0.786	8
VM 37	L - TGTCCGCGTTCTATAAATCAGC R - CGAGGATGAAGTAACAGATGATC	$(AG)_5.(CCT)_3.(CT)_{13}$	0.808	0	0.763	6
VM 39	L - GATGGTTGTAATGGGAGAGTC R - AAAAGGATGAAATTAGGGAGCA	$(AC)_{13}.(AT)_5.(TACA)_4$	0.863	1.000	0.827	12
VM 68	L - CAAGGCATGGAAAGAAGTAAGAT R - TCGAAGCAACAAATGGTCACAC	$(GA)_{15}$	0.757	0	0.707	6
CEDG 007	L - GAAGTTGACACTCATCCACC R - GTGCAGCCACTACATGAATG	$(AG)_{16}$	-	-	-	-

Continues in the next page.

Table 4. Continued.

Primers	Sequence (5'→3')	Repeat motif	H_E	H_O	*PIC*	*NA*
CEDG 008	L - GCCCATATTTTTACGCCCAC R - AGGCGAGGTTTCGTTTCAAG	$(AG)_{26}$	0.383	0	0.358	5
CEDG 015	L - CGCCAAAGGAAACGCAGAAC R - CCCGATGAACGCTAATGCTG	$(AG)_{27}$	0	0	0	1
CEDG 024	L - TTTGGTGAAGATGACAGCCC R - CATCTTCCTCACCTGCATTC	$(AG)_{18}$	0.529	0	0.422	3
CEDG 026	L - TGGGACAAACCTCATGGTTG R - TCAGCAATCACTCATGTGGG	$(AG)_{16}$	0	0	0	1
CEDG 043	L - ACTATTTCCAACCTGCTGGG R - AGGATTGTGGTTGGTGCATG	$(AG)_{14}$	0.545	0	0.482	4
GBssr-MB13	L - GCAGCAACAACAGCAACA R - GCAGGTTTTGTGGCTCAG	$(GAG)_4$	0	0	0	1
GBssr-MB14	L - TGGAATTTGGAAGGAAGGA R - GATGCAGGTGTTTGGGAG	$(AAGA)_4$	0.776	0.100	0.725	7
GBssr-MB17	L - ACCTGCAAGTTGGCAAGA R - TATGTGCACGCATGGAAG	$(AG)_{10}$	0	0	0	1
GBssr-MB77	L - GGAGAGGAAGGAACAGGG R - GGCAGAGCATAACATGGC	$(GTT)_5(GA)_5A(AG)_6$	0.554	0.739	0.493	7
GBssr-MB91	L - GAGGCCAATCCCATAACTTT R - AGCACCACATCAGAGATTCC	$(AG)_{34}(GA)_{14}$	0	0	0	1
Mean	-	-	0.507	0.149	0.468	4.95±3.22

H_O, observed heterozygosity; H_E, expected heterozygosity; *PIC*, Polymorphism Information Content; *NA*, number of allele.

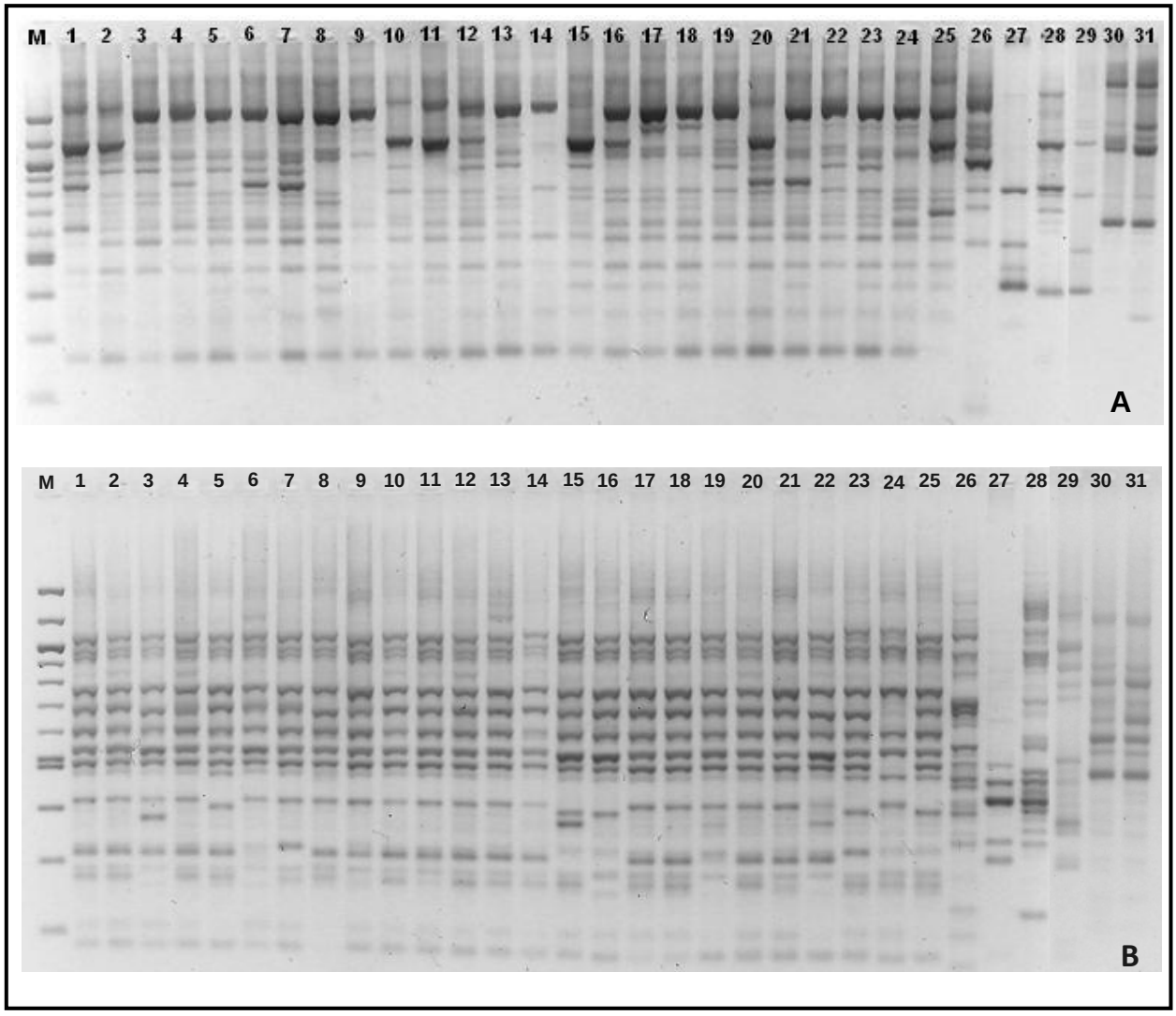

Figure 1. The generated fragments in 1,2 % agarose gel **A)** DAF fragments obtained for 31 accessions with the primer OP-G6 after electrophoresis in agarose gel. **B)** The ISSR polymorphism using the ISSR primer 888. **M:** 100 bp ladder molecular weight marker; **1:** *Vigna unguiculata* 'Pitiuba'; **2:** *V.ung*., Epace10; **3:** *V.ung.*, Vita; **4:** *V.ung*., IPA201; **5:** *V.ung*., Vita5; **6:** *V.ung*. 'Sempre Verde'; **7:** *V.ung*. 'Canapu Amarelo'; **8:** *V.ung*. 'Pérola'; **9:** *V.ung*., BR17-Gurguéia; **10:** *V.ung*., IPA204; **11:** *V.ung*., IPA205; **12:** *V.ung*., IPA206; **13:** *V.ung*., CNC0434; **14:** *V.ung*., BR14-Mulato; **15:** *V.ung*., IT85F-2687; **16:** *V.ung*. IT86D-716-1; **17:** *V. ung*., TVU382; **18:** *V. ung*., TVU379; **19:** *V.ung*., TE96.282.22G; **20:** *V.ung*., CE31; **21:** *V.ung*., CE315; **22:** *V.ung*., BR9 Longá; **23:** *V.ung*., IT81D-1053; **24:** *V.ung.* ssp. *cylindrica*; **25:***V.ung.* ssp. *sesquipedalis*; **26:** *V. angularis*; **27:** *V. radiata*; **28:** *V. mungo*; **29:** *V. aconitifolia*; **30:** *Phaseolus vulgari*s 'Neckar'; **31:** *P. vulgari*s 'Delinel'.

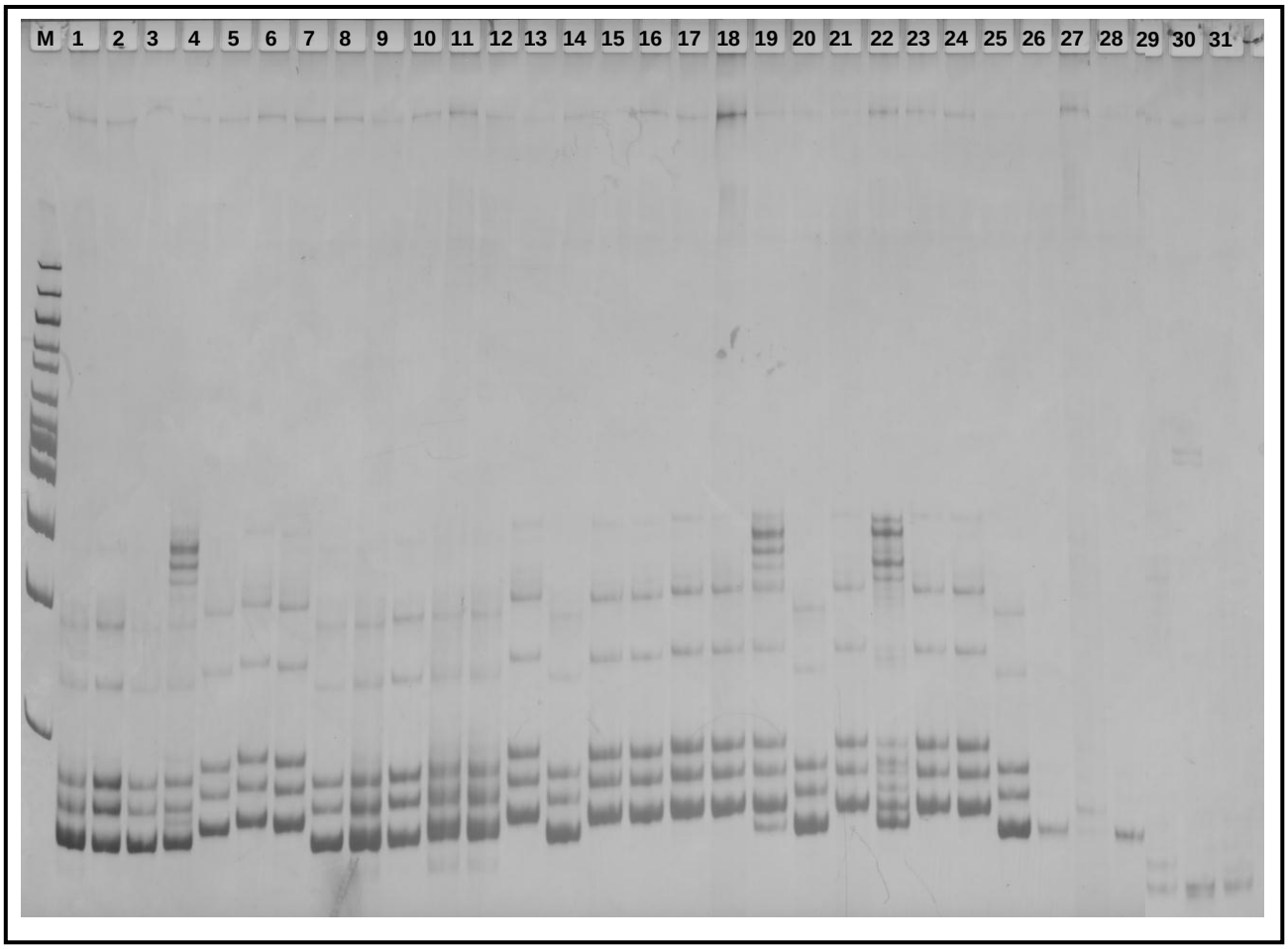

Figure 2. SSR polymorphisms using the primer pair VM36. **M:** 100 bp ladder molecular weight marker. **1:** *Vigna unguiculata* 'Pitiuba'; **2:** *V.ung*., Epace10; **3:** *V.ung.* 'Vita'; **4:** *V.ung*., IPA201; **5:** *V.ung*., Vita5; **6:** *V.ung*.-Sempre Verde; **7:** *V.ung.* 'Canapu Amarelo'; **8:** *V.ung.* 'Pérola'; **9:** *V.ung*., BR17-Gurguéia; **10:** *V.ung*., IPA204; **11:** *V.ung*., IPA205; **12:** *V.ung*., IPA206; **13:** *V.ung*., CNC0434; **14:** *V.ung*., BR14-Mulato; **15:** *V.ung*., T85F-2687; **16:** *V.ung*., IT86D-716-1; **17:** *V.ung*., TVU382; **18:** *V.ung*., TVU379; **19:** *V.ung*., TE96.282.22G; **20:** *V.ung*., CE31; **21:** *V.ung*., CE315; **22:** *V.ung*., BR9-Longá; **23:** *V.ung*., IT81D-1053; **24:** *V.ung.* ssp. *cylindrica*; **25:** *V.ung.* ssp. *sesquipedalis*; **26:** *V. angularis*; **27:** *V. radiata*; **28:** *V. mungo*; **29:** *V. aconitifolia*; **30:** *Phaseolus vulgaris* 'Neckar'; **31:** *P. vulgaris* 'Delinel'.

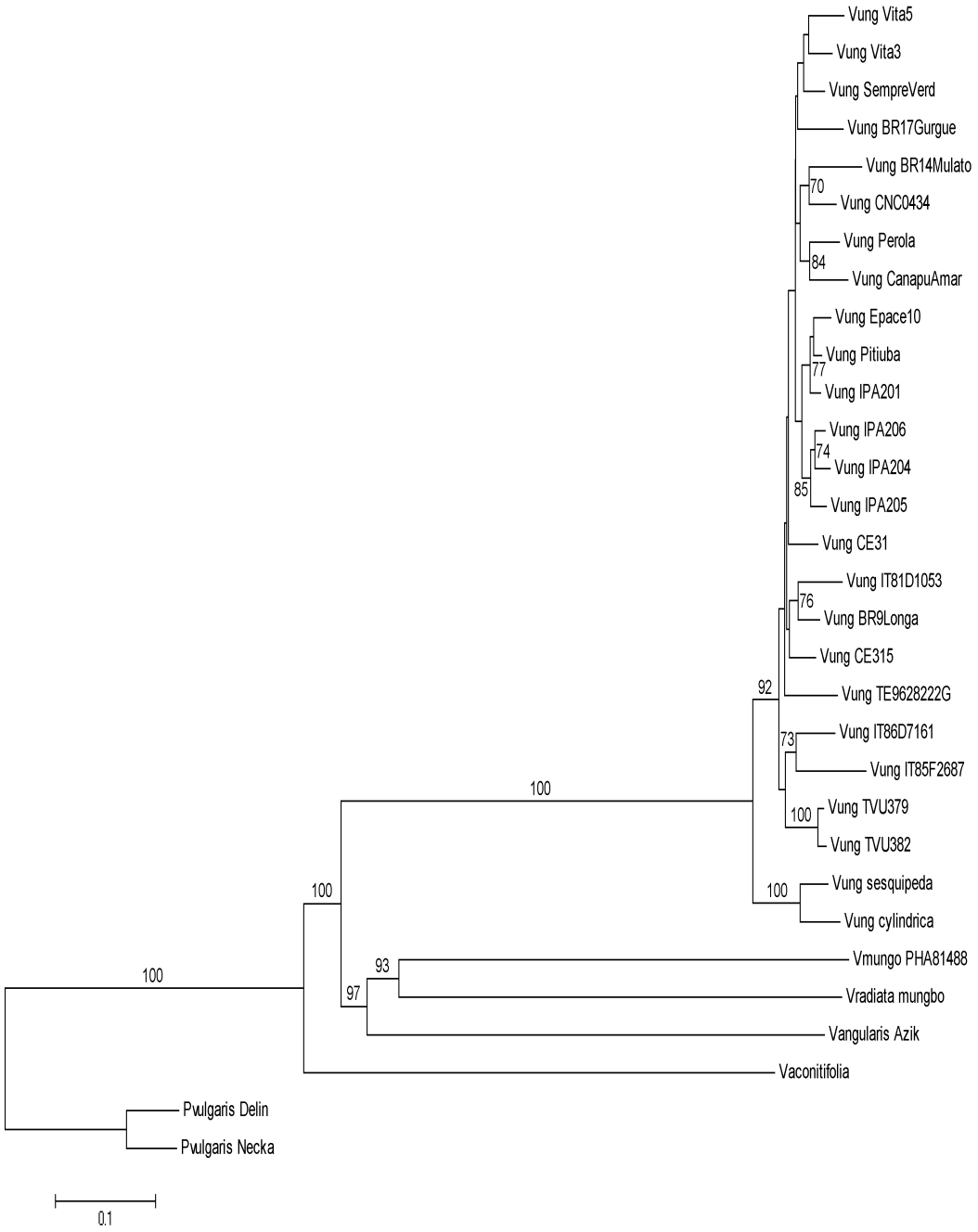

Figure 3. Phenogram using neighbor-joining, showing relationships among *Vigna* species and *Phaseolus* outgroup combining DAF and ISSR data. Numbers in the base of the branches regard to bootstrap values (≥50%) for 1,000 replications.

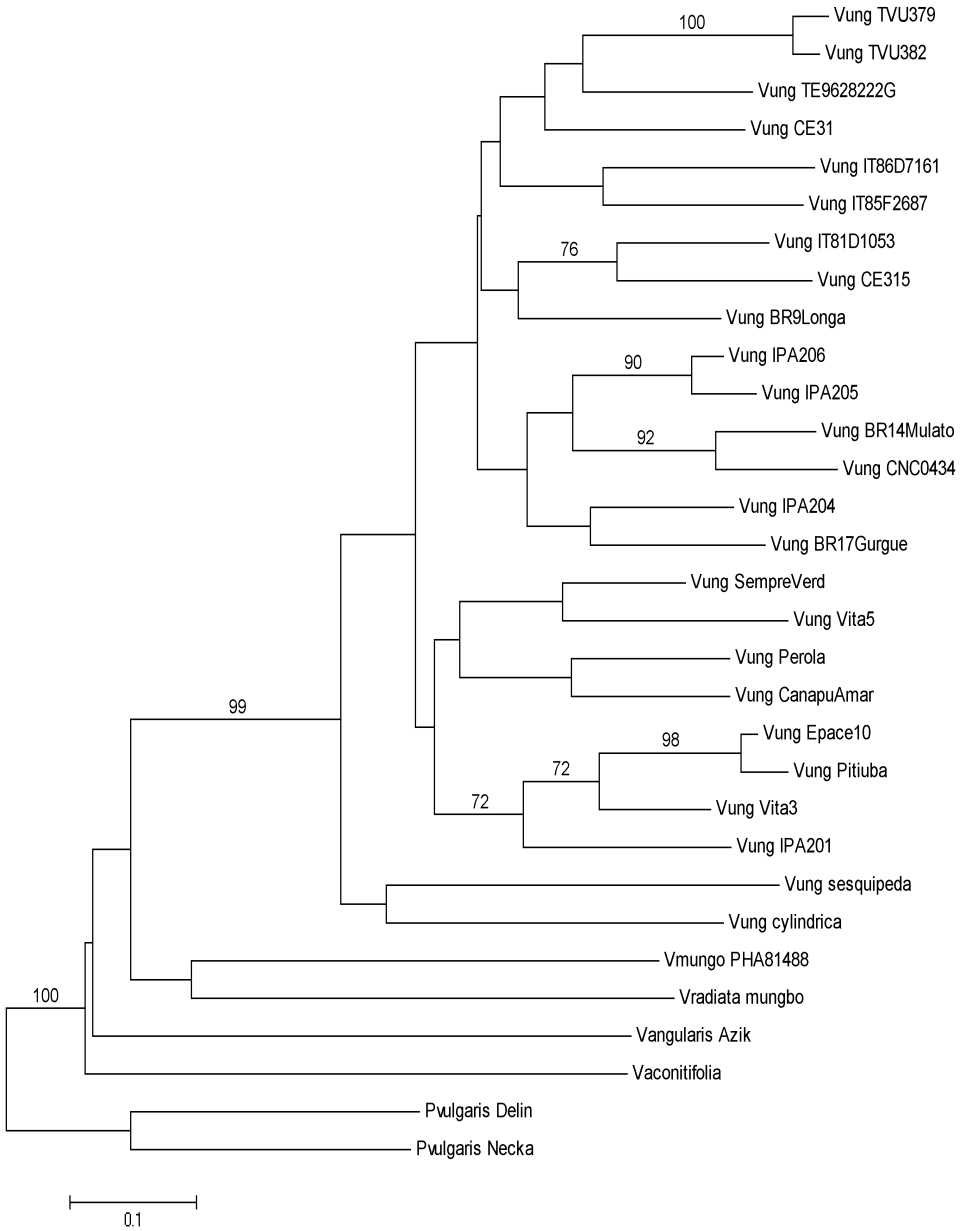

Figure 4. Phenogram showing relationships among *Vigna* species and *Phaseolus* outgroup, using SSR markers based on neighbor-joining. Numbers in the base of the branches regard to bootstrap values (≥50%) for 1,000 replications.

6. CAPÍTULO 2

APPLICABILITY OF DAF AND ISSR MARKERS FOR POLYMORPHISM DETECTION AMONG COWPEA PARENTAL CANDIDATES FOR MAPPING PURPOSES*

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ABSTRACT

Cowpea [*Vigna unguiculata* (L.) Walp.] is an important crop which provides essential nutrients and high quality protein for human and livestock feed in several tropical countries, being also recognized as a potential source of useful genes for legume breeding. Despite the great potential of this crop, information on genetic diversity and relationships among cowpea genotypes from Brazil is still very limited. This kind of information is necessary for cowpea breeding programs for the acquisition of high yield and resistance to major disease-pest complexes and environmental stresses. To allow the accomplishment of various objectives regarding cowpea genetic improvement, as well as to conserve the existing genetic resources of the species, this study aimed to evaluate the applicability of DAF and ISSR markers in the characterization of four cowpea candidates for mapping purposes, a necessary approach, especially considering the narrow genetic basis of the species. Using DAF analysis, 650 fragments could be scored, of which 190 were polymorphic, with an average of 4.3 polymorphic fragments per primer. Out of 89 ISSR primers evaluated in the study, 57 primers amplified 491 fragments. From these, 42 primers generated 113 polymorphic bands, with an average of 2.6 polymorphic fragments per primer, while 32 produced no clear polymorphism, or generated only faint bands. Mean of polymorphism information content (PIC) for each marker system (0.11 for DAF and 0.08 for ISSR) suggested that both marker systems were equally effective in determining polymorphisms among sampled genotypes. Thus, taking into account the low number of accessions available, the results observed were enough for diversity evaluation. Furthermore, the identification of molecular markers is essential for mapping approach and breeding programs, especially when it comes to crops with low amount of information about genetic diversity as cowpea. Consequently, the described set of ISSR and DAF markers will be highly useful not only for genetic mapping, but also for genetic diversity studies with wild and cultivated *Vigna* species.

Keywords: *Vigna unguiculata*, marker screening, molecular profile, DAF, ISSR.

1. Introduction

Cowpea [*Vigna unguiculata* (L.) Walp.], a member of the Fabaceae family, is part of traditional cropping systems in the semi-arid regions of tropical countries, being used for various purposes such as for food, fodder for livestock and fixation of atmospheric nitrogen. The estimated area under cowpea cultivation worldwide is about 14 million ha, with more than 4.5 million tons of annual production (Singh *et al.* 2003), of which approximately 70% is concentrated in three countries: Nigeria, Niger and Brazil (Singh *et al*. 2002).

Great progress has been achieved in cowpea breeding programs and a wide range of varieties has been developed by the International Institute of Tropical Agriculture (IITA) and agencies in the United States and Brazil, combining diverse plant types with high yield potentials (Freire-Filho *et al.* 2005). However, only few of these high yielding varieties are resistant to biotic and abiotic stress. Thus, a major goal of cowpea breeding and genetic improvement programs is combining desirable agronomic traits (e.g. drought and salt tolerance) to resistance against viruses, fungal pathogens, root-knot nematodes and insects (Sawadogo *et al.* 2010; Timko and Singh, 2008).

An important aspect for genetic improvement of cowpea, regarding tolerance against different kinds of stress, is the availability of genetic diversity in the germplasm collection, allowing the selection of favorable genes and alleles for incorporation into the breeding populations (Asare *et al*. 2010). Conventional diversity analysis methods in the field are time and resources consuming, laborious and drastically affected by environmental factors. Therefore, a rapid technique which is not affected by environmental changes is needed for the genetic diversity assessment and parental lines selection to use in hybrid development programs. The assessment of genetic variability using molecular markers appears to be an attractive alternative to conventional diversity analyses and can also aid both management and conservation of biodiversity (Gajera *et al.* 2010).

In recent years, significant progress on molecular characterization and functional analysis of important genes has been achieved with different molecular approaches in cowpea and its related species. Data obtained by Xavier *et al.* (2005) and Fang et al. (2007), for instance, pointed out to the relatively low level of polymorphism of random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) markers in cowpea cultivars. Despite that, Simon *et al.* (2007) observed a somewhat high level of polymorphism using DNA amplification fingerprinting (DAF) markers, which was successfully applied for a phylogenetic analysis among cowpea cultivars, with emphasis to domesticated accessions from Brazil,

including remnants from accessions introduced by African slaves and colonizers during the XVI century.

Aiming to generate a genetic map segregating for important qualitative and quantitative traits under tropical conditions, two crosses have been considered: BR 14-Mulato x IT85F-2687 and IT86D-716.1 x Tvu-382, in both cases including a Brazilian accession (available at Embrapa germplasm) versus an African (IITA) accession, presenting very promising contrasting features among the progenitors, with emphasis on the resistance to two very important cowpea viruses: CPSMV (*Cowpea Severe Mosaic Virus*) and CABMV (*Cowpea Aphid-borne Mosaic Virus*).

Thus, we studied here the applicability of DAF and ISSR markers to characterize cowpea cultivars used in breeding programs aiming resistance to virus infections and mapping approaches.

2. Materials and methods

Genomic DNA was isolated from young leaves of four accessions (BR 14-Mulato, IT85F-2687, IT86D-716.1 and Tvu-382), following the standard CTAB protocol (Weising *et al.* 1995) with modifications described by Benko-Iseppon *et al.* (2003). Contaminating polysaccharides were selectively precipitated (Michaels *et al.* 1994) and DNA concentrations were determined by electrophoresis in 1.2 % agarose gel using known amounts of phage λ-DNA (MBI, Fermentas, Hanover, MD, USA) as standard.

ISSR analysis included anchored and unanchored primers (UBC Primer Set no. 9, University of British Columbia, Canada). The ISSR amplifications were carried out in 20 μL reactions containing 15 ng of genomic DNA, 1× reaction buffer, 2.5 mM $MgCl_2$, 200 μM of each dNTP, 50 pmol primer and 0.7 U Taq DNA polymerase (Fermentas). For each primer, a gradient PCR ranging from 45 °C to 65 °C was used to determine the optimum annealing temperature. The PCR cycling conditions were as follows: denaturation at 94 °C for 4 min and 35 cycles of 30 s at 94 °C, 45 s with varied temperatures as per the melting temperature of the ISSR primers used, 2 min at 72 °C and 7 min final extension step at 72 °C.

A total of 52 DAF oligonucleotides with arbitrary sequences were evaluated in this study. The DAF reactions followed the protocol described by Simon *et al.* (2007), with 1 ng of genomic DNA, 1.5 μL 10× PCR buffer, 2.5 mM $MgCl_2$, 10 mM dNTP-mix, 50 pmol primer and 0.7 U Taq DNA polymerase (Fermentas), adjusted to the final volume of 15 μL with bi-distilled sterile H_2O. The DNA was first denatured for 2 min at 95 °C, followed by 40 cycles of 15 s denaturation at 95 °C; 1 min annealing at 35 °C and 2 min extension at 72 °C, with a final

extension at the same temperature for 2 min. Both DAF and ISSR reaction products were separated by electrophoresis on ethidium bromide-stained 1.8% agarose gels in 0.5× TBE buffer at 70 V for 4 hours. The amplicons were observed under UV light and then photographed. The gel was also compared with the Gene Ruler 100 bp DNA Ladder Plus (Fermentas).

After polymorphism analysis, two matrixes were constructed based on polymorphisms for each band/individual regarding the evaluation for the presence (1) or absence (0) of each band or amplicon. Polymorphic information content (PIC) values were calculated for each DAF and ISSR primer according to Roldán-Ruiz et al. (2000): $PIC_i = 2\ f_i\ (1 - f_i)$, where PIC_i is the polymorphism information content marker i, f_i the frequency of present marker fragments and $1 - f_i$ the frequency of absent marker fragments.

3. Results and Discussion

Obtained results from the evaluation using ISSR primers are shown in Table 1. Thirty two primers did not produce any fragments, or generated only faint non-reproducible bands. From these primers, fifteen were constituted by an AT/TA core motif (including one with a TAT motif), seven by CT/TC motifs, three by GT repeats and eight by tri, four and five-nucleotide repeats. Souframanien and Gopalakrishna (2004) reported a similar situation for black gram (*Vigna mungo*), despite the fact that AT/TA dinucleotide-repeats are thought to be the most abundant in plant species (Langercrantz *et al.* 1993). A possible explanation of these results is that ISSR primers based on AT motifs are self-annealing due to sequence complementarity, thus allowing the formation of primer dimers during PCR amplifications (Blair et al. 1999). Furthermore, the lower annealing temperature of these AT-rich primers should be considered as an explanation for the lack of fragment amplification using relatively high annealing temperatures (Kumar *et al.* 2009).

Of the 57 ISSR primers that resulted in successful amplification, 15 did not reveal polymorphisms among the analyzed cowpea parental lines. The number of amplified fragments per primer ranged from 1 to 19 with an average of 8.6, with the number of polymorphic bands ranging from 1 to 8 with an average of 1.9. The average number of polymorphic bands detected in our approach was lower than those obtained in previous studies on the genetic diversity of *Vigna*. In particular, Ghalmi *et al.* (2010) have reported an average of 5.4 polymorphic bands per primer in cowpea landraces. Nevertheless, their study showing genetic polymorphism was based on the use of pre-screened highly-informative primers in the genus *Vigna* provided by Ajibade *et al.* (2000) and Souframanien and Gopalakrishna (2004). To the best of our knowledge, this is the

first report using a large number of ISSR primers in cowpea cultivated genotypes. Additionally, our results are similar to those reported by Gupta et al. (2008), which 21 ISSR were found to be polymorphic in parental lines and mapping populations of black gram with an average of 1.9 polymorphic loci per primer.

The range of PIC values of ISSR primers ranged from 0.00 (monomorphic bands) to 0.29 (UBC-808), with an average of 0.08, which were lower than previously found for black gram and mungbean (*Vigna radiata*) by Tantasawat *et al.* (2010a). However, these authors used a high-resolution ISSR analysis with polyacrylamide gels, thus increasing the number of scorable polymorphic markers. Similarly, Pharmawati et al. (2005) showed that the type of gel electrophoresis and staining method used may influence the number of scored bands and thus affect the level of polymorphism observed. However, in contrast to our results for cowpea, in chickpea (*Cicer arietinum*) 56.2% of polymorphism was detected among cultivated varieties with six ISSR primers (Rao *et al.* 2007), while in faba bean (*Vicia faba* L.) populations, 98.9% of polymorphism was observed with four primers (Terzopoulos and Bebeli, 2008). Thus, the lower PIC values found for cowpea may be resulted from the utilization of closely related accessions, as observed by Tantasawat *et al.* (2010b) in asparagus bean, also reflecting the narrow genetic basis considering available breeding material.

Among the used primers, UBC-842, 850 and 849 (PIC values 0.229, 0.215 and 0.208, respectively) were the most informative in distinguishing cowpea accessions. It is remarkable that these primers have GA and GT repeat motifs in their sequences, since polymorphism based on GT-rich ISSR primers had not been reported so far among cowpea accessions. Previous studies reported high polymorphism only by using GA, AG and CA-based ISSR primers among cowpea genotypes (Ajidabe *et al.* 2000; Ghalmi *et al.* 2010). Thus, it is clear that the potential in generating polymorphic fragments among accessions by using ISSR markers depends on the variety and frequency of SSRs and their distribution along the analyzed genome (Morgante and Olivieri 1993; Depeiges et al., 1995).

After ISSR primer screening, five primer combinations (UBC-807/841, UBC-808/857, UBC-811/857, UBC-810/857 and UBC-811/807) were selected, based on the number of amplified fragments per primer combination. Regarding the primer UBC-811, the use of a single primer generated only one monomorphic band in all tested accessions, while its combination with primer UBC-807 amplified, four were polymorphic, revealing the potential of ISSR primers combination, when a single primer is not informative. The generation of extra variability by using ISSR primers combination may therefore enhance the ability of the technique to distinguish among closely related genotypes and, at the same time, reduce the need for screening

a large number of primers. Furthermore, the ability to produce additional amplicons from different loci would greatly enhance the value of the technique for mapping purposes (Cekic *et al.* 2001).

The DAF markers resulted in a higher number of amplified fragments than ISSR markers. In total, 52 primers produced 635 bands of which 190 were polymorphic. The total number of scored bands per primer ranged from 5 (OPK06) to 22 (OPD12) with an average of 12.2. OPF02/OPK02 and OPB07/OPG08 amplified the minimum and maximum number of polymorphic fragments per primer which were 1 and 8, respectively with an average of 3.6. The PIC scored per primer varied from 0.00 to 0.29 with an average of 0.12 (Table 2).

Studies on the discriminatory power of DAF primers have not been carried out in cowpea so far, but similar studies considering PIC values have been done with ISSR and SSR markers. Li *et al.* (2001), for instance, reported PIC values ranging from 0.02 to 0.73 with a mean of 0.47 among cultivated cowpea genotypes, whereas Gioi et al. (2010) observed PIC varying between 0.30 and 0.72 (0.54 on average) among 48 wild cowpea lines. This difference is not surprising because codominant markers are usually more accurate than dominant markers in detecting polymorphism levels among genotypes (Guillot *et al.* 2011). Tantasawat *et al.* (2010a), for instance, observed that PIC averages from SSR markers were higher than those from ISSR. In contrast, ISSR also provided a better assessment than SSR of the genetic relatedness among *Vigna* genotypes. Remarkably, Laurentin and Karlovsky (2007) revealed the lack of consistency between either the number of fingerprints of sesame (*Sesamum indicum*) elite lines with exclusive fingerprints suggesting that it would be better to consider how many genotypes are discriminated by a primer, instead of calculating parameters such as PIC, RP (resolving power) and MI (marker index).

As literature including genetic polymorphism of Brazilian cowpea accession is still very scarce, the present study could help the researchers in this regard in future.

Acknowledgments

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Table 1. Primer sequences, amplified bands, polymorphic bands and PIC values in ISSR analysis (ordered from higher to lower PIC values).

Primer	Repeat	T_a (ºC)	Amplified bands	Polymorphic bands	PIC
808	$(AG)_8C$	52.8	9	6	0.292
816	$(CA)_8T$	50.4	9	5	0.250
845	$(CT)_8RG$	54.0	3	2	0.250
842	$(GA)_8YG$	54.0	12	8	0.229
850	$(GT)_8YC$	54.0	11	6	0.216
849	$(GT)_8YA$	52.8	12	6	0.208
807 x 841	$(AG)_8T$ x $(GA)_8YC$	52.0	12	6	0.208
819	$(GT)_8A$	50.4	5	2	0.200
851	$(GT)_8YG$	54.0	9	4	0.194
869	$(GTT_6$	50.4	2	1	0.188
825	$(AC)_8T$	50.4	7	3	0.161
848	$(CA)_8RG$	54.0	8	3	0.156
836	$(AG)_8YA$	54.0	5	2	0.150
847	$(CA)_8RC$	54.0	6	2	0.146
891	$HVH(GT)_7$	52.0	16	5	0.133
808 x 857	$(AG)_8C$ x $(AC)_8YG$	52.0	16	5	0.133
811 x 857	$(GA)_8C$ x $(AC)_8YG$	52.0	16	5	0.125
810 x 857	$(GA)_8T$ x $(AC)_8YG$	52.0	13	4	0.125
809	$(AG)_8G$	52.8	11	3	0.125
844	$(CT)_8RC$	52.8	6	2	0.125
887	$DVD(TC)_7$	52.0	12	3	0.115
859	$(TG)_8RC$	54.0	10	3	0.113
864	$(ATG)_6$	51.9	10	3	0.113
888	$BDB(CA)_7$	52.0	19	5	0.112
818	$(CA)_8G$	52.8	8	2	0.109
879	$(CTTCA)_3$	49.7	14	4	0.107
811 x 807	$(GA)_8C$ x $(AG)_8T$	52.0	14	4	0.107
890	$VHV(GT)_7$	52.0	12	3	0.104
834	$(AG)_8YT$	52.8	10	2	0.100
858	$(TG)_8RT$	52.0	9	2	0.097
855	$(AC)_8YT$	52.0	10	2	0.088
884	$HBH(AG)_7$	52.0	15	3	0.083
810	$(GA)_8T$	50.4	9	2	0.083

Table 1. Continued.

Primer	Repeat	Ta (ºC)	Amplified bands	Polymorphic bands	PIC
826	$(AC)_8C$	52.8	9	2	0.083
830	$(TG)_8G$	52.8	6	1	0.083
886	$VDV(CT)_7$	52.0	13	2	0.077
841	$(GA)_8YC$	52.8	12	2	0.063
835	$(AG)_8YC$	52.8	6	1	0.063
857	$(AC)_8YG$	54.0	13	2	0.058
873	$(GACA)_4$	52.0	9	1	0.056
876	$(GATA)_4$	49.7	16	2	0.047
817	$(CA)_8A$	50.4	8	1	0.047
840	$(GA)_8YT$	52.8	8	1	0.047
862	$(AGC)_6$	58.0	8	1	0.047
878	$(GGAT)_4$	52.0	9	1	0.042
856	$(AC)_8YA$	52.0	13	1	0.038
828	$(TG)_8A$	52.8	10	1	0.038
880	$(GGAGA)_3$	49.7	12	0	0.000
889	$DBD(AC)_7$	52.0	12	0	0.000
812	$(GA)_8A$	50.4	9	0	0.000
866	$(CTC)_6$	58.0	9	0	0.000
846	$(CA)_8RT$	52.8	8	0	0.000
861	$(ACC)_6$	58.0	8	0	0.000
807	$(AG)_8T$	50.4	7	0	0.000
860	$(TG)_8RA$	52.0	4	0	0.000
824	$(TC)_8G$	51.9	3	0	0.000
868	$(GAA)_6$	49.7	3	0	0.000
829	$(TG)_8C$	52.8	2	0	0.000
853	$(TC)_8RT$	52.0	2	0	0.000
811	$(GA)_8C$	52.8	1	0	0.000
827	$(AC)_8G$	52.8	1	0	0.000
852	$(TC)_8RA$	52.0	1	0	0.000

Table 2. Primer sequences, amplified bands, polymorphic bands and PIC values considering DAF results.

Primer	Sequence (5'-3')	Amplified bands	Polymorphic bands	PIC
OPB-7	GGTGACGCAG	11	8	0.295
OPH-5	AGTCGTCCCC	11	8	0.284
R26010	GACCGACACG	8	5	0.281
OPI-5	TGTTCCACGG	10	7	0.275
OPK-4	CCGCCCAAAC	12	7	0.239
OPG-8	TCACGTCCAC	14	8	0.232
OPW-16	CAGCCTACCA	14	7	0.223
OPJ-12	CCACACTACC	14	7	0.214
OPH-1	GGTCGGAGAA	7	4	0.214
OPN-7	CAGCCCAGAG	11	5	0.204
OPC-2	GTGAGGCGTC	15	7	0.191
OPM-14	AGGGTCGTTC	14	7	0.187
15-12	AGGTCTTGGGTAGGC	12	6	0.187
OPL-17	AGCCTGAGCC	15	7	0.183
OPG-6	GTGCCTAACC	18	7	0.173
15-17	TCTCCGCAACGCAAC	16	6	0.164
OPJ-16	CTGCTTAGGG	8	3	0.156
OPK-14	CCCGCTACAC	13	5	0.144
OPJ-13	CCACACTACC	14	5	0.142
OPA-3	AGTCAGCCAC	7	2	0.142
OPG-4	AGCGTGTCTG	11	4	0.136
15-3	TGCGTGCTTGAGAGA	14	4	0.125
OPB-9	TGGGGGACTC	15	5	0.125
OPE-12	TTATCGCCCC	15	4	0.125
OPE-19	ACGGCGTATG	9	3	0.125
OPD-13	GGGGTGACGA	18	5	0.118
OPG-12	CAGCTCACGA	16	4	0.109
15-1	TGCGTGCTTGTATAA	15	4	0.108
R4602	GCAGGATACG	7	2	0.107

Table 2. Continued.

Primer	Sequence (5'-3')	Amplified bands	Polymorphic bands	PIC
R47010	CGCAGACCTC	7	2	0.107
15-19	CTATGCCGACCCGAC	16	4	0.101
OPE-06	AAGACCCCTC	14	3	0.098
OPB-17	AGGGAACGAG	13	3	0.096
OPF-10	GGAAGCTTGG	11	2	0.079
OPD-12	CACCGTATCC	22	4	0.073
OPE-03	CCAGATGCAC	14	2	0.071
OPX-11	GGAGCCTCAG	11	2	0.068
OPB-11	GCGAAAGCCAA	14	2	0.062
HP-B-III	GCGACAGCAGA	12	2	0.062
OPK-02	GTCTCCGCAA	6	1	0.062
AIV	GCGAAAGCCAA	15	2	0.058
OPC-09	CTCACCGTCC	13	2	0.057
OPL-03	CCAGCAGCTT	13	2	0.057
OPF-02	GAGGATCCCT	11	1	0.034
OPC-06	GAACGGACTC	14	0	0.000
OPJ-19	GGACACCACT	12	0	0.000
OPA-18	AGGTGACCGT	11	0	0.000
OPB-05	TGCGCCCTTC	11	0	0.000
OPD-08	GTGTGCCCCA	10	0	0.000
OPI-01	ACCTGGACAC	10	0	0.000
OPA-13	CAGCACCCAC	06	0	0.000
OPK-06	CACCTTTCCC	05	0	0.000

7. CAPÍTULO 3

A GENETIC LINKAGE MAP FOR COWPEA [*Vigna unguiculata* (L.) WALP.]

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ARTIGO A SER SUBMETIDO À REVISTA *MOLECULAR BREEDING*

ABSTRACT

Genetic maps are the most effective approach to connect molecular data with breeding strategies. Until today, all cowpea genetic maps available were based in crosses among Californian and African cultivars and, although they brought many advances, these were not able to cover some important features necessary to improve the yield of this crop, especially in tropical regions. The present study aimed to generate and saturate the first genetic map based on a segregating population consisted of 93 RILs (Recombinant Inbred Lines) derived from a cross between two breeding lines, the Brazilian elite "BR 14-Mulato" and the IITA elite "IT85F-2687", which segregated to important biotic and abiotic features according to the Brazilian Cowpea Breeding Program. The linkage map was constructed using the Mapmaker 2.0 software, with a LOD score of 3.0 and a recombination frequency of 0.35. The current map includes 216 loci, mapped into eleven linkage groups (LGs), corresponding to the haploid chromosome number of cowpea. 98 loci were detected by DAF (DNA Amplification Fingerprinting), 73 loci by SSR (Simple Sequence Repeats), 24 loci by ISSR (Inter Simple Sequence Repeat) and 21 loci by AFLP (Amplified Fragment Length Polymorphism) markers. The overall map length was 2,064.6 cM, with an average distance of markers of 9.7 cM. The length of each linkage group ranged from 68.8 (LG11) to 323.3 cM (LG1). The results obtained will support the basis for the development of specific saturated maps for studies in quantitative traits to be used in cowpea programs, as well as to comparison studies with integration to linkage maps of other *Vigna* species. The developed markers will be very useful for isolation of genes involved in resistance or tolerance against biotic and abiotic stresses via map-based cloning.

Key-words: cowpea, genetic map, molecular makers, breeding program.

1. Introduction

Genetic mapping associated with molecular markers is an important approach in biological research, constituting a prerequisite for plant molecular breeding and crop improvement (Yang et al. 2011). An increased knowledge of the structure and composition of the genomic structure in legumes can be provided by genetic maps using contrasting parental accessions, with potential to help in the stacking of desirable agronomic traits, such as those governing abiotic stress tolerance (e.g. drought, salinity), plant architecture redesign and improvement of the resistance or tolerance against biotic stresses, including bacterial, fungal, viral diseases, insects and nematodes (Freire Filho et al. 2005; Singh 2005; Timko et al. 2007).

The genus *Vigna* is an outstanding group within the Fabaceae family, including morphologically highly variable species that occur in tropical, subtropical and temperate regions (Onyilagha et al. 2008). Among existing taxa, a number of economically important crop species occurs, such as cowpea [*Vigna unguiculata* (L.) Walp.], black gram [*V. mungo* (L.) Hepper], mungbean [*V. radiata* (L) Hepper], azuki bean [*V. angularis* (Willd.) Ohwi and Ohashi], rice bean [*V. umbellata* (Thunb.) Ohwi and Ohashi] and moth bean [*V. aconitifolia* (Jacq.) Maréchal] (Tomooka et al. 2002).

Cowpea is a staple food of great economic and social importance, especially in developing countries, particularly in regions as West and Central Africa, parts of South America (particularly North-Eastern Brazil and Peru) and parts of South Asia. In the US, the Middle East, and the Southern regions are also important cowpea producers (Ehlers and Hall 1997). In Brazil, cowpea is the main source of protein, minerals and vitamins for a significant portion of the low-income population (Freire Filho et al. 2005).

Research approaches on cowpea genomics have gained momentum due to some features present in this crop considering both, molecular and classical genetics. Key attributes of cowpea include diploidy ($2n = 22$) and autogamous fertilization, a small genome (620 Mb), a rapid reproductive cycle (2 to 3 months) and a high level of diversity in available cultivars (Timko et al. 2008).

Various types of molecular markers have been described for trait mapping and marker-assisted selection in cowpea, such as RAPD (Randomly Amplified Polymorphic DNA), DAF (DNA Amplification Fingerprinting), AFLP (Amplified Fragment Length Polymorphism), SCAR (Sequence Characterized Amplified Regions), ISSR (Inter Simple Sequence Repeat) and SSR (Simple Sequence Repeats). According to Muchero et al. (2009a) access to most of the genes in cowpea can be gained through cDNA sequences, which represent expressed genes.

Partial cDNA sequences, known as ESTs (Expressed Sequence Tags), facilitate the identification of SNPs in protein-encoding genes, allowing also the development of microsatellite markers, both useful in the generation of genetic linkage maps that represent a gene-based framework of the genome.

There are 183,000 EST at the HarvEST database, as a result from a project carried out by the University of California Riverside and IITA-Generation Challenge Program (GCP) project. Additionally, over 250,000 gene-space sequence reads (GSRs), with an average length of 610 bp, were also generated by sequencing and analysis of the gene-rich, hypomethylated portion of the cowpea genome (Chen et al. 2007; Timko et al. 2008). Xu et al. (2010) identified 1,010 SSR marker (410 eSSR and 600 GSS-SSR), from unigene sequences downloaded from the University of California-Riverside HarvEST and gene-space sequences with homology to known genes (Chen et al. 2007) downloaded from the University of Virginia CGKB database.

Altogether, three genetic maps of cowpea have been constructed by Menancio-Hautea et al. (1993), Menendez et al. (1997) and Ouédraogo et al. (2002). Biochemical and phenotypic agronomical traits have also been located on the genetic map by Ouédraogo (2002). Recently, a consensus genetic map of cowpea was constructed based on 928 EST-derived SNP markers and a specific map for seedling drought stress-induced premature senescence, providing a solid basis for gene/QTL mapping, especially for features such as resistance against disease, insects and yield under drought stress, besides comparative genomic studies (Muchero et al. 2009a; 2009b). However, the cowpea consensus map may not fulfil the needs of the Brazilian cowpea breeding program as well as other similar tropical regions, considering particular traits of interest. Therefore, the objective of this study was to construct a cowpea linkage map using molecular markers applied in a F_6-F_7 segregating population from a cross among two contrasting accessions BR14-Mulato (Brazilian program) and IT85F-2687 (IITA program), which segregate to important biotic and abiotic features desirable to the Brazilian Cowpea Breeding Program.

2. Material and Methods

Plant materials and DNA extraction

A F_6-F_7 population consisted of 93 recombinant inbred lines (RILs) was derived from a cross between two agronomically contrasting breeding lines, the Brazilian elite "BR 14-Mulato" and the IITA elite "IT85F-2687" (Table 1).

Table 1. Morphological and agronomic traits scored in the parental lines.

Trait	BR14-Mulato	IT85F-2587
Growth habits	undetermined	undetermined
Plant size	prostrate	prostrate
Type of leaf	globular	semi lanceolate
Flower color	purple	white
Immature pod color	green	green
Color of harvest pods at maturity	yellow	purple
Pod length	20 cm	16,5 cm
Number of seeds per pod	17	16
Seed shape	rhomboid	reniform
Seed coat color	brown	white
Weight of 100 grains	17 g	12 g
Number of days to full flowering	45 - 55	65 - 70
Life cycle (in days)	65 - 75	65 - 70
Response to Cowpea severe mosaic virus (CPSMV)	resistant	susceptible
Response to Cowpea aphid-borne mosaic virus (CPAMV)	susceptible	resistant
Response to Cowpea golden mosaic virus	highly resistant	highly resistant
Response to *Erysiphe polygoni*	moderately resistant	moderately resistant

DNA was isolated from expanding leaves of three-week old plants using a modified CTAB (cetyl-trimethyl-amoniumbromide) protocol (Weising et al. 1995), followed by RNAse treatment and precipitation of contaminating polysaccharides (Michaels et al. 1994). DNA concentrations were determined electrophoretically in agarose gel 1.2% comparing the fluorescence intensities using known amounts of phage λ-DNA as a reference.

DAF Analysis

A total of 105 DAF primers were screened between the two cowpea parental accessions, including decamers from Operon Technologies, and also self developed 11- and 15-mers. The DAF reactions followed Simon et al. (2007) with minor modifications [2 ηg of template, 1.5 μl 10x PCR buffer, 1.5 mM $MgCl_2$, 2.5 mM dNTP-mix (MBI Fermentas), 50 pmol primer e 0.5 U *Taq* DNA polymerase (MBI Fermentas), adjusted to a final volume of 15 μl with bidestilated sterile H_2O]. The amplification reaction was carried out in a TC-412 Techne® Thermal Cycler. The DNA was first denatured for 2 min at 95ºC, followed by 40 cycles of 15 s denaturation at 95ºC; 1 min annealing at 35ºC and 2 min elongation at 72ºC, with a final elongation at the same temperature for 2 min. The DAF reaction products were separated on 1.8% agarose gels at 70 V for 3 h, stained with ethidium bromide, revealed and photographed under ultraviolet light.

ISSR Analysis

A total of 30 ISSR primers from the #9 ISSR primer kit of the Biotechnology Laboratory, University of British Columbia (UBC, Vancouver, Canada) were applied, after pre-selection by Amorim et al. (unpublished data). DNA amplifications were performed in a 15 μl reaction volume containing approximately 20 ng template DNA, 50 μM of a single primer UBC, 200 μM of each dNTPs and 0,5 U of Taq DNA polymerase (MBI Fermentas) in 1× PCR buffer, and 0.4 mM $MgCl_2$.

Amplification was performed in thermal cycler with an initial denaturation at 94ºC for 10 min, followed by 35 cycles of 94ºC for 30 s, 49,7 up to 58ºC (depending on the primer) for 35 s and 72ºC for 2 min, followed by final extension at 72ºC for 7 min, being finally stored at 4ºC. The products were electrophoresed on a 1.8% agarose gel at 70 W for 3 h, stained with ethidium bromide (0.5 mg/ml) and photographed.

SSR Analysis

A total of 220 microsatellite primer pairs (SSRs) from six different sources were applied in the parental lines for polymorphism screening, including: 110 cowpea SSR primer pairs previously mapped on asparagus bean (Xu et al. 2011), 11 cowpea SSR primer pairs (Li et al. 2001), 33 azuki bean SSR primer pairs mapped on the azuki bean linkage map (Han et al. 2005), one mungbean SSR primer (Gwag et al. 2006), 35 primers from *Phaseolus vulgaris* (PV series) and tree markers from the BM (Bean microsatellite), both (PV and PM) according to Garcia et al. (2011). Additionally, we developed 27 SSR markers after data mining on EST data from Cowpea NordEST database, TIGR Plant Transcript Assemblies and Cowpea Genomics Knowledge Base. Perfect SSR motifs were identified with software TRA (Bilgen et al. 2004) and used for primer design with aid of the program Primer3 (Rozen and Skaletsky 2000).

SSR amplifications were performed in 20 µl reactions containing 30 ng template DNA, 0.5-10 µM of each primer, 200 µM of dNTPs, 0.5 U of Taq DNA polymerase (Fermentas) in 1× PCR buffer and 0.4 mM $MgCl_2$. PCR amplifications were carried out using a TC-412 Techne® Thermal Cycler. Depending on the SSR primer pair used, the PCR schedule was adapted according to the primer sources, as follows: (1) For SSR by Xu et al. (2011): 94°C for 3 min, followed by 36 cycles of 94°C for 20 s, 52°C for 30 s, 72°C for 40 s, and a final extension for 5 min at 72°C; (2) For SSR by Li et al. (2001): 93°C for 5 min, followed by 38 cycles of 94°C for 1 min, 54 to 60°C for 30 s depending on primers used, 72°C for 1 min, and a final extension for 5 min at 72°C; (3) For SSRs developed by Han et al. (2005): 94°C for 2 min, followed by 35 cycles of 94°C for 15 s, 55°C for 15 s, 68°C for 5 min and non-labelled primers; (4) For SSR by Gracia et al. (2011): 94°C for 5 min, followed by 30 cycles at 94°C for 1 min, the specific annealing temperature of the primer for 1 min, 72°C for 1 min, and a final extension for 7 min at 72°C. (5) For the SSR primers developed on the present study: 95°C for 5 min followed by 30 cycles of 95°C for 1 min, 54°C for 1 min, 72°C for 1 min, and a final extension for 10 min at 72°C.

The generated SSR fragments were separated in 5% non-denaturing polyacrylamide gels and were visualized after silver nitrate staining, according to Creste et al. (2001).

AFLP Analysis

AFLP analysis was performed using AFLP analysis system I kit (Invitrogen Corp., Carlsbad, CA, USA) following the manufacturer's instructions. The 64 possible primer combinations were tested in cowpea parental accessions. Denatured selective amplification products were run on 7% denaturing polyacrylamide gel at 65 V for 2.5 h in 1× TBE buffer. After silver nitrate staining (Bassam et al. 1991), gels were dried at room temperature and photographed. The polymorphic bands were named according to the name of each primer pair and numbered serially in descending order of fragment size.

LEG Marker Analysis

Twenty-two Legume anchor markers reported by Hougaard et al. (2008) were evaluated in both parents. These markers are indicated as 'Leg primers' or "mtmt_gen" and were developed by György Kiss's group in Gödöllõ, Hungary, for the Grain Legume Integrated Project (GLIP). After the amplification, sequence polymorphisms (SNPs) between the parental accessions were used to develop CAPS markers.

Linkage analysis and distribution of markers along the map

Genetic mapping segregation data, derived from a matrix based on polymorphic bands from the different markers applied to the RIL population (including DAF, SSR, AFLP, ISSR and LEG/CAPS evaluations) were used to create a genetic map. It was based on linkage analysis with MapMaker software (v.2.0) for Windows (Lander et al. 1987), three-point analysis with a minimum LOD of 3.0 and maximum recombination fraction (θ) of 0.35. The Kosambi function was used to convert recombination frequencies into map distances (cM; Kosambi 1944). Then, the "first order" command was used to determine a linear order. The remaining loci in each group were then placed on the "try" command and were then considered as accessory markers. The ordered marker sequences were confirmed by the "ripple" command. Linkage maps were drawn manually in excel sheet. For each segregating marker, a $\chi 2$ goodness-of-fit analysis was performed to test for deviation from the 1:1 expected segregation ratio at a 5% level of significance.

3. Results

Polymorphism and markers for mapping purposes

Results considering the generation and features of polymorphic bands are described on Table 2 and 3.

The analysis of 105 DAF markers performed among the parental accessions (BR14-Mulato x IT85F-2687) showed 90 (85.7%) polymorphic markers and 15 (14.3%) monomorphic bands. In total, 82 DAF primers were used for genotyping the RIL population and 107 polymorphic bands were used for linkage map construction. ISSR analysis showed 24 (80 %) polymorphic markers and six (20%) monomorphic markers. In total, 24 ISSR primers were used for genotyping the RIL population and 35 polymorphic bands were used for linkage map construction.

After screening of 110 SSR primer pairs (named CLM markers) developed by Xu et al. (2011), 72 (62.5%) accessed polymorphic and 38 (34.5%) monomorphic loci. Among these, 41 polymorphic primer pairs were used in the RIL population, resulting in 43 segregating bands used to linkage map construction. All SSR cowpea primer pairs created by Li et al. (2001) amplified fragments, but only four revealed scorable polymorphisms and three were used for mapping (VM35, VM36 and VM68). All SSR functional primers developed in this study were able to reveal scorable polymorphisms.

For SSR markers transferable, of the 33 SSR primer pairs screened from azuki bean, 26 (78.8%) were transferable and 10 (38.4%) showed polymorphisms among the parents. From these, seven (CEDG008, CEDG036, CEDG043, CEDG044, CEDG111, CEDG214 and CEDG271) were used in the RIL population for mapping. The only mungbean SSR primer polymorphic was GBssr-MB8g, also used for mapping. Of the 38 SSR primer pairs tested from common bean, 28 (73.6%) amplified successfully, but only eight of the PV series revealed polymorphisms between the progenitor and only one (PV67) was efficient for mapping.

Results from the AFLP analysis revealed that among the 64 primer combinations tested, 61 primer pairs resulted in polymorphic bands. The number of polymorphic fragments per primer pair ranged from 8 to 13, with an average of 10 polymorphic fragments per primer. Primer pairs that generated more than ten bands between the two parental accessions were selected for application of RILs. In total, 59 polymorphic bands were used for linkage map construction.

Table 2. Number of analyzed markers and polymorphic bands used for linkage map construction.

Markers Type	Number of analyzed markers		Number of polymorphic bands	
	Between parental accessions	Used in genotyping of the RIL population	Used for linkage map construction	Loci Mapped
DAF	105 markers	82 markers	107 bands	98 loci
ISSR	30 markers	24 markers	35 bands	24 loci
SSR	220 markers	80 markers	82 bands	73 loci
AFLP	64 markers	17 markers	59 bands	21 loci
LEG	22 markers	10 markers	10 bands	-
Phenotypic	1 marker associated to CPSMV	1 marker associated to CPSMV	1 marker	-
Total of loci used for mapping			294 loci	216 loci

Table 3. Number of analyzed SSR primer pairs and polymorphic bands used for linkage map construction.

SSR Primer pairs		Number of analyzed primer pairs			Number of polymorphic bands	
Source	Screened from	Between parental accessions	Number and percentage polymorphic	Used in genotyping of the RIL population	Used for linkage map construction	Loci Mapped
Xu et al. 2011	*V. unguiculata*	110	72 (65.45%)	41	43	39
Li et al. 2001	*V. unguiculata*	11	4 (36.36 %)	3	3	2
Han et al. 2005	*V. angularis*	33 (26 were transferable)	10 (38.46%)	7	7	7
Gwag et al. 2006	*V. radiata*	1 (1 was transferable)	1 (100%)	1	1	1
Garcia et al. 2011	*Phaseolus vulgaris*	38 (28 were transferable)	8 (28.57%)	1	1	1
Derived from: Cowpea NordEST database TIGR Plant Transcript Assemblies Cowpea Genomics Knowledge Base		27	27 (100%)	27	27	23
Total		220	122 (55.45%)	80	82	73

Construction of cowpea linkage map

Among the 294 loci used for preliminary mapping in the cowpea population (107 DAF, 59 AFLPs, 82 SSRs, 35 ISSR, 10 LEG/CAPS, and one phenotypic marker associated to Cowpea severe mosaic virus, CPSMV), 216 mapped to 11 linkage groups (LGs). Total map length was 2,064.6 cM, providing an average marker density of 1 marker per 9.7 cM. In our map, market densities varied significantly, due to pronounced clustering in some regions (especially in the LGs 1, 3, 7 and 9), as observed in Figure 1 and Table 4, where map features are presented. The length of LGs varied from 68.8 cM (LG11) to 323.3 cM (LG1). The number of marker loci per LG ranged from 6 to 41. LG1 included most markers with an average marker density of 7.9 cM in length.

Table 4. Features of the cowpea genetic linkage map from the intraspecific cross among BR14-Mulato and IT85F-2687.

		Number of Polymorphisms per Marker Type					
Linkage Groups	**Length (cM)**	**Total Markers**	**DAF**	**AFLP**	**ISSR**	**SSR**	**Average distance between markers (cM)**
LG1	323.3	41	14	02	05	20	7.9
LG2	311.6	24	12	02	04	06	12.9
LG3	239.1	30	21	00	04	05	7.9
LG4	238.4	18	09	03	02	04	13.2
LG5	204.6	18	07	03	01	07	11.3
LG6	168.2	16	06	01	03	06	10.5
LG7	146.5	24	09	01	01	13	6.1
LG8	142.7	14	04	00	00	10	10.2
LG9	142.1	19	11	06	01	01	7.5
LG10	79.3	06	02	02	02	00	13.2
LG11	68.8	06	03	01	01	01	11.4
Total	2,064.6	216	98	21	24	73	9.7

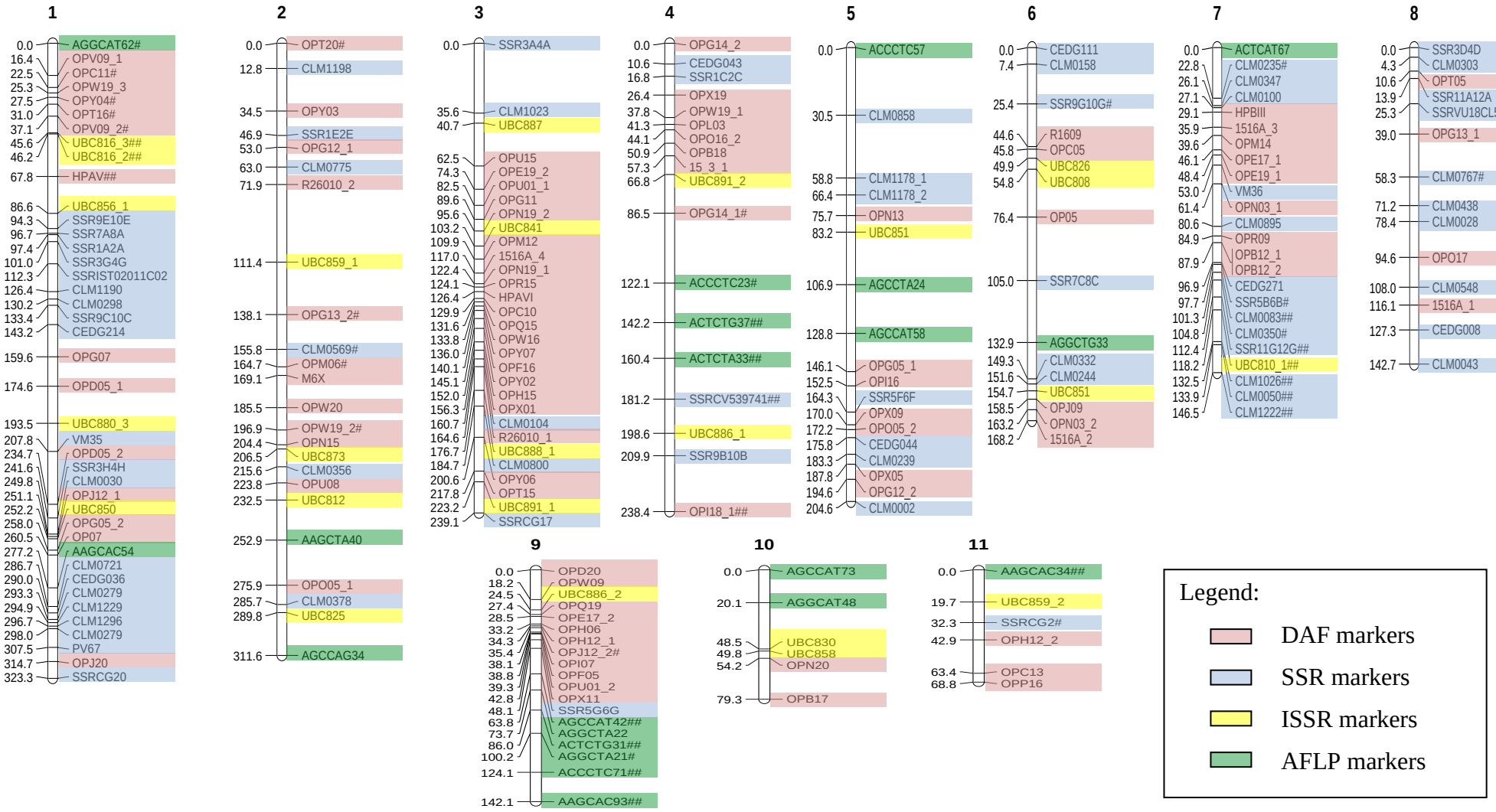

Figure 1. Genetic linkage map of cowpea (*Vigna unguiculata*) intraspecific cross based on a recombinant inbred population derived from a cross between BR14-Mulato and IT85F-2687. Cumulative recombination distances (in cM) are shown on the left and marker loci are shown on the right side of the linkage groups. Distorted markers were indicated with # ($P < 0.05$) and ## ($P < 0.01$).

4. Discussion

Despite the narrow genetic basis of domesticated cowpea, results of this study revealed that DAF technique was the most efficient for the generation of informative molecular markers. In this study, it was obtained close to one loci mapped for every DAF primers tested across parents (98/105). Menéndez et al. (1997) screened 332 RAPD primers on cowpea parental accessions (IT84S-2049 e 524B), 133 loci was mapped. So, they had about half of their proportion (133/332). Gupta et al. (2008) used 360 random primers to screen polymorphisms across *V. mungo* parental accessions. They had 86 loci mapped, which found much smaller proportion than Menéndez et al (1997), less than one loci mapped for every four RAPD primers tested across parents (86/360).

Later studies on legumes have used RAPD markers for the development of linkage maps. Primers screening on progenitors also indicated low levels of polymorphism using such markers. Tanyolac et al. (2010) reported a total of 384 RAPD primers in the parental accessions of *Lens culinaris* to detect polymorphisms. Of the 384 RAPD primers screened, 116 primers yielded 192 segregating bands (30.2%). Blair et al. (2012) evaluated a total of 698 RAPD primers in a *Phaseolus vulgaris* cross (DOR364 x BAT477). From these, only 104 markers were useful for genetic mapping, based on 59 decamer primers.

Considering SSR markers, the level of polymorphisms in cowpea has been increased, as in the case of Sawagodo et al. (2010) and Asare et al. (2010), which identified polymorphisms ranging from 76.3% to 97% among cowpea accessions, respectively, using such markers.

Also Xu et al. (2010) developed 1,010 SSR markers (410 eSSR and 600 GSS) using cowpea sequences. A later approach by the same group (Xu et al. 2011) expanded the utility of these markers with a new set of primers and amplified 1,326 SSR (494 eSSRs, 632 GSSs, 200 GSSs derived from BACs), applied to a cross using two asparagus bean accessions (*V. unguiculata* ssp. *sesquipedalis*) for mapping purposes. An overall technical success rate of 92.3% was achieved, allowing the detection of 210 polymorphic loci that provided an average polymorphic rate of 16.5%. A total of 184 SSR markers were allocated to linkage groups. This map helped us to choose among the most informative and appropriate SSR markers for transferability screening to cowpea. Thus, a significantly higher percentage of polymorphic markers was identified and transferred to our map in this study. Considering the results, a future goal for the cowpea map shall include the association of each SSR polymorphic marker in cowpea with asparagus bean map, providing information on the genetic relationships among these related subspecies.

The number of primers generating polymorphic azuki bean SSR markers (38.4%) was lower than that reported by different researchers in *Vigna* species. Gupta et al. (2008) used 55 azuki SSR primer pairs to screen polymorphism among cultivated and wild black gram parents. Of these, 45 primer pairs (81.8%) revealed polymorphisms. Aoyama et al. (2011) reported 122 azuki bean SRR primer pairs and observed higher numbers of polymorphisms in azuki bean parental lines. These authors used cross combinations between the dwarf mutant (ad1) and several wild-type plant representatives. The combination using primers ad1 and Acc2482 exhibited higher polymorphism (63.9%), than observed a combination between ad1 and Acc2265 (41.8%). Besides the fact that azuki bean and black gram are related species (Simon et al. 2007), the azuki SSR analysis has been performed using a fluorescent fragment detection system (Aoyama et al. 2011). The lower number of informative markers obtained in this study may be justified by the use of the silver nitrate staining method, since it is less sensitive in polymorphism detection.

The majority of the polymorphic SSR markers fit the expected 1:1 segregation. However, segregation of 14 markers (19.1% of total) significantly deviated from this ratio ($P \leq 0.05$). The AFLP markers revealed high level segregation distortion (47.6%) when compared with the 12.2% observed for DAF markers, 12.5% for ISSR and 19.1% for SSR markers. Besides LEG/CAPS markers had no marker positioned on the map, AFLP showed lower number of markers allocated to eleven individual cowpea linkage groups.

The preliminary linkage map generated in the present study identified 11 linkage groups, in agreement with 11 haploid chromosomes of cowpea ($2n = 22$). A significant amount of markers (78) could not be placed on the map, indicating that several areas of the genome remain undetected, considering the present set of available markers. Similar situations have been observed in other crosses, what is justified by the fact that genetic maps with good genome coverage and confidence in locus order require large numbers of DNA markers (Semagn et al. 2006).

Some previously published AFLP and SSR linkage maps also showed clustering of these markers in centromeric or telomeric regions, associated with the low-copy fraction of plant genomes, probably due to an excess of repeats in this area and suppressed recombination shrinking the genetic map relative to the DNA content (Jeuken et al. 2001; Morgante et al. 2002). In the present study it was observed that AFLP and SSR prevailing on centromeric regions for LG1 and in the telomeric region for LG7. Chromosome centromeric regions are usually conserved and may not be polymorphic when self-pollinated plants are evaluated. This is in accordance with the observed clustering of markers mapped on some intraspecific segregating

crosses (Saliba-Colombani et al. 2000; Truco et al. 2007). In contrast, random markers (DAF or RAPD) were generally more evenly distributed throughout the genome (Diaz et al. 2011), similarly to this study, where DAF revealed wide distribution and in few cases also appeared connected in clusters (as in LG3).

The inbred lines evaluated by Menendez et al. (1997) and Ouédraogo et al. (2002) were used in an initial saturated genome mapping work, being this last map based on a mixture of molecular markers and phenotypic traits for disease and insect resistance.

The genetic Brazilian map of cowpea is now comparable in marker density with previous studies. Analyzing each chromosome individually, the extensions were also greater. Besides that, the map showed a lower average interval between markers (9.7 cM), comparing with the 6.43 cM and 0.73 cM, reported by Ouédraogo et al. (2002) and Muchero et al. (2009a), respectively. The higher level of saturation in the recent consensus map was justified by the types and number of markers used.

Two other approaches have included QTL mapping directly linked to disease resistance and insect pest. First, Omo-Ikerodah et al. (2008) used 92 RILs from a cross between susceptible and resistant lines to identify genetic loci associated with the expression of resistance to flower bud thrips. Secondly, Muchero et al. (2011) used a consensus genetic linkage map, incorporating SNP markers from the IT93K-503-1 × CB46 map and five other RIL populations for the delayed maturity related to senescence traits and *Macrophomina* resistance loci in cowpea.

A key feature regarding future studies of cowpea map and QTL analysis should consider conserved synteny between related species and model legumes. Comparative mapping analyses have focused primarily on *V. radiata*, *V. mungo* and *V. umbellata* (Chatieng et al. 2006; Somta et al. 2008; Isemura et al. 2010), and also on the legumes *Glycine max* and *Medicago truncatula* (Muchero et al. 2009a). The present study reports the development of the first genetic map for cultivated cowpea including SSR markers screened from data banks, as well as published ones. Considering the narrow genetic basis, most promising levels of polymorphism observed in the present evaluation and earlier studies, emphasizes the need to use and develop additional polymorphic SSR markers. So, genetic maps with high marker density can be developed in future.

5. Conclusion

The results obtained in this work establish the basis for gene/QTL mapping that control important phenotypic traits, such as resistance to disease, pests and genome characterization. Furthermore, an increase in the marker density with functionally expressed genes may provide the necessary tools needed for the marker assisted selection.

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8. CONCLUSÕES

- Os marcadores DAF, ISSR e SSR foram eficientes tanto na distinção de diferentes espécies do gênero *Vigna*, como entre os acessos de feijão-caupi, por gerar frequências polimórficas elevadas nos níveis intraespecífico e interespecífico;

- As taxas de transferabilidade dos marcadores SSR, desenhados para *V. angularis*, *V. radiata* e *Phaseolus vulgaris*, foram elevadas, o que possibilita a aplicação desses marcadores nos estudos comparativos de diversidade genética e de mapeamento em diferentes espécies de *Vigna*;

- Os fenogramas, gerados a partir dos marcadores dominantes (DAF e ISSR) e codominantes (SSR), revelaram agrupamentos de acordo com a taxonomia. As cultivares de feijão comum (*Phaseolus vulgaris* ssp. *vulgaris*) tiveram comportamento adequado como grupo externo, enquanto as espécies asiáticas de *Vigna* posicionaram-se basalmente aos acessos de *V. unguiculata*, sendo *V. aconitifolia* na posição basal, comportando-se como grupo irmão;

- Os dados moleculares apresentados (DAF, SSR e ISSR) e agronômicos (resistência/tolerância e susceptibilidade/sensibilidade a estresse biótico e abiótico) apresentaram boa correspondência na formação dos grupamentos nas árvores genéticas. Propiciando desta forma maior confiabilidade na escolha dos parentais e desenvolvimento de mapas genéticos e para a definição de cruzamentos voltados ao melhoramento;

- Os marcadores SSR, DAF, ISSR e AFLP mostraram-se úteis na obtenção do mapa genético. O bom nível de polimorfismo e distribuição por todo o genoma, citados em outros trabalhos, foi confirmado. Desta forma, foi possível obter um mapa com nível médio de saturação. Porém, sugere-se a realização de trabalhos futuros visando propiciar uma maior cobertura e a localização de regiões associadas com o vírus do mosaico severo do feijão-caupi (CPSMV) e o vírus transmitido por afídeos (CABMV);

- A proporção entre número de marcadores analisados entre os parentais e número de loci gerados no mapa foi elevada quando comparada com a literatura.

- Este estudo apresenta uma relevante contribuição para o melhoramento e estudo do genoma do feijão-caupi, utilizando marcadores SSR já disponíveis para outras espécies do gênero *Vigna* permitindo novos estudos de sintenia;

- Os marcadores e o mapa gerados indicam haver grande potencial para o melhoramento associado a seleção assistida por marcadores em feijão-caupi no Brasil, especialmente considerando a diversidade e riqueza dos bancos genéticos disponíveis, revelada no presente estudo.

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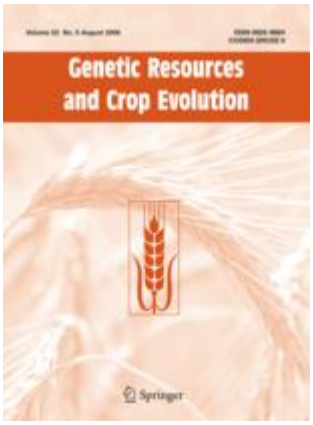

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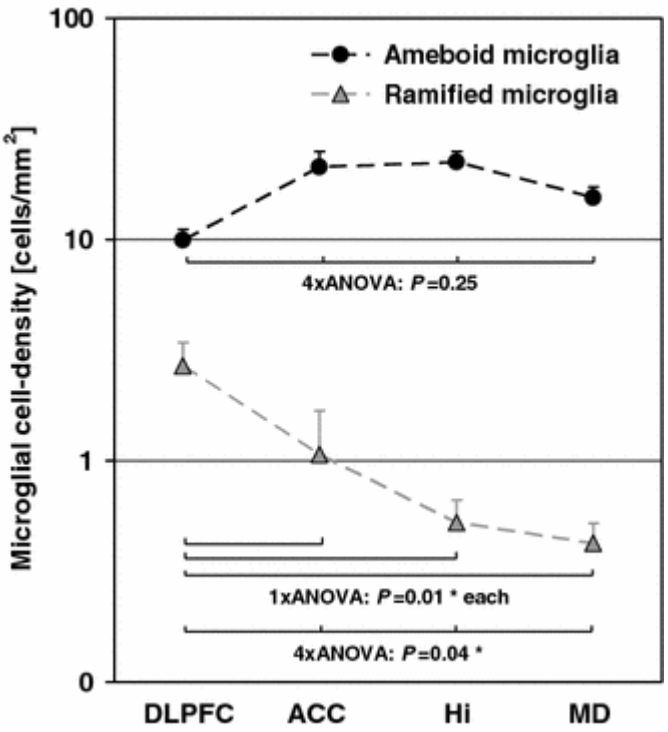

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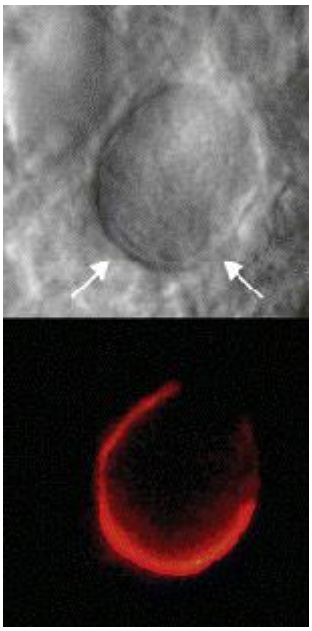

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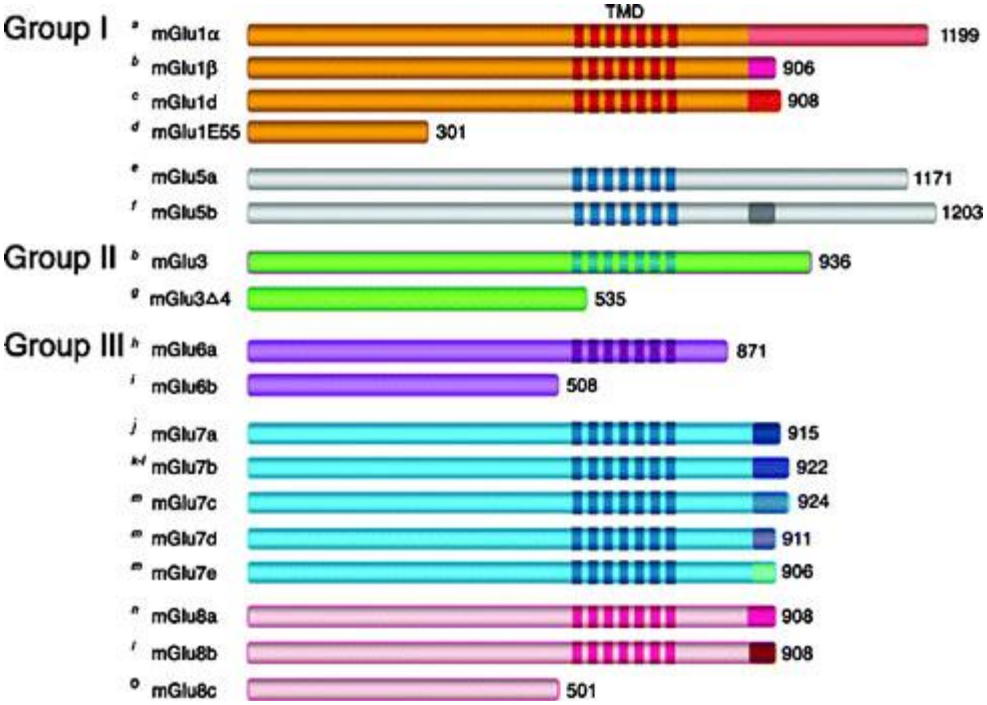

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