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ANÁLISE ESTRUTURAL E FUNCIONAL DO GENOMA DO FEIJÃO-CAUPI: MAPA GENÉTICO E ELEMENTOS TRANSPONÍVEIS

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Tese apresentada ao curso de Doutorado do Programa de Pós Graduação em Ciências Biológicas, da Universidade Federal de Pernambuco, como parte dos requisitos para obtenção do título de Doutor em Ciências Biológicas, na área de concentração Biotecnologia/Biologia Celular e Molecular.

Orientador(a): Prof[a] Dr[a] Ana Maria Benko Iseppon

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Dedico

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"O que está por trás de nós e o que está diante de nós são questões minúsculas se comparadas ao que está dentro de nós."

Ralph Waldo Emerson

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LISTA DE ABREVIATURAS

AFLP	*Amplified Fragment Length Polymorphism*; Polimorfismo de Comprimento de Fragmentos Amplificados
BAC	*Bacterial Artificial Chromosome*; Cromossomo Artificial de Bactéria
BLAST	*Basic Local Alignment Search Tool*; Ferramenta Básica de Busca por Alinhamento Local
CAPS	*Cleaved Amplified Polymorphic Sequence*; Polimorfismo de Sequência Amplificada e Clivada
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
cDNA	*Complementary DNA*; DNA Complementar
cM	*CentiMorgan*
CitEST	Projeto de Sequência Expressas de Citros
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CMA_3	Cromomicina A_3
CoffeaEST	Projeto Genoma Brasileiro Café
CONAB	Companhia Nacional de Abastecimento
CPAMN	Centro de Pesquisa Agropecuária do Meio-Norte
Cowpea HarvEST	Banco de Dados de EST do feijão-caupi da Universidade de Harvard
CTAB	*Cetyl-trimethyl-amoniumbromide*
DAF	*DNA Amplification Fingerprinting*; Impressão Genômica da Amplificação do DNA
DarTs	*Diversity Arrays Technology* ; Tecnologia de Arranjos de Diversidade
DAPI	4',6-diamidino-2-fenilindol
DNA	*Desoxyribonucleic Acid;* Ácido Desoxirribonucleico
DNAr	DNA ribossomal
DNAr 45S	DNA ribossomal 45S

DNAr 5S	DNA ribossomal 5S
DNAr 18S	Segmento 18S da Unidade de DNA ribossomal 45S
DNAr 5,8S	Segmento 5,8S da Unidade de DNA ribossomal 45S
DNAr 26S	Segmento 26S da Unidade de DNA ribossomal 45S
EMBRAPA	Empresa Brasileira de Pesquisa Agropecuária
EST	Expressed Sequence Tag; Sequências de Etiquetas Expressas
eSSR	EST derived SSR Abreviatura de EST-SSR: marcadores SSR derivados de ESTs
Facepe	Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco
FAO	*Food and Agriculture Organization*; Organização das Nações Unidas para Agricultura e Alimentação
Fapemig	Fundação de Amparo à Pesquisa do Estado de Minas Gerais
Fapesp	Fundação de Amparo à Pesquisa do Estado de São Paulo
Finep	Financiadora de Estudos e Projetos
FISH	*Fluorescent In Situ Hybridization*; Hibridização *In Situ* Fluorescente
FORESTs	Projeto do Transcriptoma do Eucalipto (https://forests.esalq.usp.br/)
GenBank	Gene Bank (http://www.ncbi.nlm.nih.gov/Genbank/); Banco de Genes
GENOLYPTUS	Rede Brasileira de Pesquisa do Genoma do *Eucalyptus*
GENOSOJA	*Soybean Genome Project*; Projeto do Genoma da Soja
IITA	*International Institute of Tropical Agriculture*; Instituto Internacional de Agricultura Tropical
IPA	Instituto Agronômico de Pernambuco
ISSR	*Inter Simple Sequence Repeats*; Região Interna de Sequências Simples Repetidas
ITS	*Internal Transcribed Spacer*; Espaçador intergênico
LG	*Linkage Group*; Grupo de Ligação
LOD	*Logarithm of an Odds score*; Logaritmo da Probabilidade
Mb	Mega pares de bases = 1.000.000 bp (bp, do inglês *base pair*)

MEGA	*Molecular Evolutionary Genetics Analysis*; Análises Genéticas da Evolução Molecular
Microarray	*DNA Microarray;* Microarranjo de DNA
MPSS	*Massively Parallel Signature Sequencing*; Sequenciamento Paralelo Massivo de Assinaturas
mRNA	*Messenger RNA*; RNA Mensageiro
MW	*Molecular Weight*; Peso Molecular
NCBI	*National Center for Biotechnology Information* (http://www.ncbi.nlm.nih.gov/); Centro Nacional para Informação Biotecnológica
NordEST	Projeto Brasileiro de Pesquisa do Genoma do Feijão-caupi
ORF	*Open Reading Frame*; Quadro de Leitura Aberto
PCR	*Polymerase Chain Reaction*; Reação em Cadeia da Polimerase
pI	*Isoeletric Point*; Ponto Isoelétrico
Phytozome	Banco Genômico de Plantas
QTL	*Quantitative Trait Loci*; Loci de Características Quantitativas
RACE	*Rapid Amplification of cDNA Ends*; Amplificação Rápida das Extremidades do cDNA
RAPD	*Randomly Amplified Polymorphic DNA*; DNA Polimórfico Amplificado ao Acaso
RILs	*Recombinant Inbred Lines*; Linhas Endogâmicas Recombinantes
RD	*Repression Domain*; Domínio de Repressão
RNA	*Ribonucleic Acid; Ácido Ribonucleico*
RT-qPCR	*quantitative Real Time PCR;* PCR quantitativa em Tempo Real
SAGE	*Serial Analysis of Gene Expression;* Análises em Série da Expressão Gênica
SCAR	*Sequence Characterized Amplified Region*; Sequências Amplificadas de Regiões
SFP	*Single Feature Polymorphism*; Polimorfismo de Caráter Único
SNPs	*Single Nucleotide Polymorphism*; Polimorfismo de Nucleotídeo Único
SSR	*Simple Sequence Repeat*; Repetição de Sequência Simples

SUCEST	*Sugarcane EST Project* (http://sucest.lad.dcc.unicamp.br/en/); Projeto EST da Cana-de-açúcar
TAIR	*The Arabidopsis Information Resource*; O Recurso de Informação sobre *Arabidopsis*
TGI	*The Gene Index Project* (http://compbio.dfci.harvard.edu/tgi/); O Projeto de Índice de Genes
Ty1-copia	*Superfamília de retrotransposon LTR*
Ty3-gypsy	*Superfamília de retrotransposon LTR*
UFPE	Universidade Federal de Pernambuco
UPGMA	*Unweighted Pair Group Method with Arithmetic Means*; Método não polarizado de Agrupamentos aos Pares com Médias Aritméticas
WGS	*Whole Genome Shotgun*; Fragmentação do Genoma Inteiro

RESUMO

O feijão-caupi é uma das principais culturas alimentares de subsistência no planeta tendo, no Brasil, maior importância nas regiões Norte e Nordeste, pela sua adaptabilidade a condições edafoclimáticas estressantes. Apresenta destacada importância social na agricultura familiar destas regiões, sendo notadamente a principal fonte de proteína para as populações de áreas semiáridas do Brasil e do norte da África. Apesar de sua rusticidade e capacidade de crescer onde outras leguminosas não se adaptam, a produtividade desta cultura pode ser afetada por fatores abióticos (como seca e salinidade) e bióticos, com ênfase para as viroses no caso do Brasil. Neste país, o melhoramento do feijão-caupi baseia-se até o momento em técnicas convencionais, havendo poucos estudos efetivamente associados às técnicas moleculares modernas, supondo-se que ferramentas moleculares possam ajudar na superação dessas adversidades. Neste contexto, o projeto Brasileiro do Transcriptoma do Feijão-Caupi (rede NordEST) gerou um número significativo de transcritos, os quais começam a ser aplicados no sentido de reverter este cenário. Adicionalmente, neste trabalho foi desenvolvido um mapa genético do genoma do feijão-caupi, onde a versão atual inclui 237 loci mapeados em doze grupos de ligação (GL), correspondentes ao número haploide do feijão-caupi. Além disso, foram realizadas análises bioinformáticas envolvendo elementos transponíveis (TEs) de forma comparativa entre soja e feijão-caupi, revelando uma diversidade significativa desses elementos em cada táxon ou entre as citadas espécies. Tais polimorfismos figuram muitas vezes associados a genes, mostrando um potencial para o desenvolvimento de marcadores moleculares. No conjunto, os dados gerados servirão como base para o desenvolvimento de estratégias visando ao conhecimento da diversidade genética e de seu potencial para o melhoramento na cultura do feijão-caupi pela associação com características fenotípicas de interesse agronômico. Novos marcadores estão sendo obtidos para cobrir melhor o genoma, devendo ser úteis em experimentos *in vitro* e *in vivo* visando ao melhoramento genético de leguminosas de interesse econômico.

Palavras-chave: feijão-caupi, marcadores moleculares, mapeamento genético, elementos transponíveis, melhoramento genético.

ABSTRACT

Cowpea is one of the main subsistence food crops of the world, whereas in Brazil it is mainly important for the North and Northeast regions due to its adaptability to stressing edaphoclimatic conditions. Its social importance deserves mentioning as the main subsistence crop and protein source of the Brazilian and North African semiarid regions. Despite of its rusticity and capacity to grow where other legumes do not adapt, the productivity of this culture may be affected by abiotic (drought and salinity) and biotic stresses, whereas with emphasis to viruses for this last type considering the case of Brazil. Cowpea breeding has been mainly based on conventional methods, whereas effectively few studies have been associated with modern molecular techniques, even though molecular tools can help to overcome these adversities. In this context, the Brazilian Cowpea Transcriptome project (NordEST network) generated a significant number of transcripts, which are beginning to be applied to change this scenario. Moreover, a cowpea genetic map is being developed, where the current version includes 237 mapped loci in twelve linkage groups (LG), corresponding to the haploid number of cowpea. Furthermore, a bioinformatic evaluation was performed involving transposable elements (TE) comparing soybean and cowpea, revealing a significant number of polymorphic loci within and among these legumes. Such polymorphisms are often associated with genes, showing a potential for the development of molecular markers. Taken together, the data generated will serve as a basis for developing strategies aiming to understand the genetic diversity and its potential for cowpea improvement through association with phenotypic traits of agronomic interest. New markers are being developed for a better coverage of the genome and should be useful for in vitro and in vivo experiments applicable to genetic improvement of economic important legume species.

Keywords: cowpea, molecular markers, genetic mapping, transposable elements, genetic breeding.

INTRODUÇÃO

O feijão-caupi [*Vigna unguiculata* (L.) Walp.] pertence à família Fabaceae (Leguminosae) e destaca-se mundialmente como um produto agrícola de alta expressão econômica e nutricional, sendo um dos grãos mais consumidos em países dos continentes africano, asiático e americano. É uma cultura versátil e de fácil manejo, com ciclo de desenvolvimento rápido, tolerância ao estresse hídrico, baixa exigência nutricional e boa capacidade de adaptação a diferentes tipos de solo, além de um importante gerador de empregos e renda (Santana *et al.*, 2007; Xavier *et al.*, 2012).

A maior parte da área plantada (86,75%) e da produção (86,5%) de feijão-caupi advêm da África, sendo a Nigéria e Níger os maiores produtores africanos e mundiais. A América contribui com 11,85% e 10,39% da área e produção, respectivamente, onde o Brasil figura como o maior produtor e o terceiro mundial (Freire Filho *et al*., 2011a). Freire Filho et al. (2011b) estimaram o percentual de participação do feijão-caupi na produção total do Brasilno período de 2005 a 2009. Neste período, a área cultivada correspondeu em média a 33,08% da área total do feijão (feijão-comum + feijão-caupi) na região Norte; a 60,80%, na região Nordeste e a 18,05% na região Centro-Oeste. Verificaram ainda que noa produção de feijão-caupi correspondeu a 37,64% na região Norte; 45,67%, na região Nordeste; e a 9,12% na região Centro-Oeste. A produtividade dos grãos foi correspondente a 113% da produtividade de feijão da região Norte (feijão-comum + feijão-caupi); a 75,3%, na região Nordeste, e a 53,26%, na região Centro-Oeste. Em termos de Brasil, no período considerado, o feijão-caupi contribuiu em média com cerca de 37,53% da área colhida, 15,48% da produção e 42,20% da produtividade total do Brasil. Isso mostra que a produção do feijão-caupi está distribuída em atividades de pequenos, médios e grandes produtores rurais, com perceptível aumento nos investimentos tecnológicos em suas áreas de produção e maior atenção dos programas de melhoramento (Freire Filho *et al*., 2011a).

O melhoramento genético convencional, por meio da seleção e de cruzamentos, objetiva aumentar a cada geração a frequência de genes associados às características fenotípicas favoráveis (Jangarelli *et al*., 2010). Desse modo, embora sendo um trabalho complexo, o desenvolvimento de cultivares altamente produtivas, precoces, de porte ereto, de crescimento determinado, resistentes a pragas e doenças, tolerantes à seca e à salinidade, estão entre os principais objetivos do melhoramento do feijão-caupi (Machado *et al*., 2008). A identificação de novas cultivares que atendam às necessidades

e à demanda dos agricultores e dos consumidores do mercado nacional e internacional envolve atividades de pesquisa que requerem muita dedicação e, sobretudo, continuidade.

O avanço contínuo das técnicas de biologia molecular, aliado à disponibilidade de sequências genômicas estruturais e funcionais, têm viabilizado o surgimento de novas metodologias para a geração e a análise de dados em genotipagem e expressão gênica a uma velocidade, precisão e escala sem precedentes (Carneiro, 2011). Ferramentas e recursos, tais como ESTs (Etiquetas de Sequências Expressas ou *Expressed Sequence Tags*) e SAGE (Análise Serial da Expressão Gênica ou *Serial Analysis of Genes Expression*), bem como a identificação de marcadores moleculares para mapeamento, têm sido desenvolvidos e melhorados para acelerar a interpretação do genoma do feijão-caupi, principalmente nos Estados Unidos, na África e no Brasil (Timko *et al.*, 2008; Wanderley-Nogueira *et al*., 2010; Kido *et al*., 2011; Soares-Cavalcanti *et al.*, 2011; Gupta *et al*., 2012).

O Consórcio Brasileiro de Pesquisa do Genoma do Feijão-caupi (rede NordEST) teve início em 2005, com o objetivo de identificar características genéticas que possam facilitar o processo de melhoramento da cultura, com foco nos diversos estresses que acometem a produção nacional, como a ocorrência de seca, altas concentrações de sais no solo e o ataque de pragas, bem como de doenças como o vírus do mosaico severo (CPSMV, *Cowpea Severe Mosaic Virus*) e potyvírus (CPAMV, *Cowpea Aphid-Borne Mosaic Virus*). Além da iniciativa que integra a Rede NordEST, destaca-se uma iniciativa desenvolvida pelas Universidades de Virgínia e da Califórnia (EUA), com sequências já disponibilizadas em bancos de dados públicos, dentro do projeto HarvEST (Cowpea HarvEST Database, Banco de Dados de EST do feijão-caupi da Universidade de Harvard), tendo gerado cerca de 180.000 ESTs (Close, 2007; Harvest, 2011).

Atualmente a rede brasileira desenvolve atividades referentes à análise e categorização das bibliotecas de ESTs (sequências do banco NordEST e dos bancos públicos HarvEST e GenBank) compiladas em um banco de dados de acesso restrito (http://bioinfo03.ibi.unicamp.br/vigna/) e a análise das bibliotecas de HT-SuperSAGE (*High Troughput Super Serial Analysis of Gene Expression*) desenvolvidas em colaboração com a Goethe University e a empresa GenXPro (ambas de Frankfurt am Main, Alemanha) no âmbito do projeto. Até 2010 o referido projeto contava com mais de 20 milhões de transcritos para análise, incluindo HT-SuperSAGE-tags, Long-SAGE-tags e ESTs, ultrapassando o número de 100 mil transcritos inicialmente planejados (Barbosa, 2010; Benko-Iseppon *et al.*, 2010; Kido *et al*., 2011).

As informações geradas no projeto NordEST podem ter múltiplas aplicações, tais como: a identificação de microssatélites e de polimorfismos de sequencias únicas (SNPs, do inglês *Single Nucleotide Polymorphism*), visando ao desenvolvimento de marcadores para mapeamento genético e localização de locos de herança quantitativa ou QTLs; a identificação de genes e famílias gênicas candidatas envolvidas na expressão do fenótipo de interesse; análises de sintenia a partir de genômica comparativa com plantas-modelo, incluindo leguminosas, colaborando com o entendimento da evolução dos genomas e da função, ação e regulação gênica; até a identificação de elementos transponíveis, importantes por seu papel estrutural e funcional no genoma, além de seu uso como marcadores moleculares.

Com o propósito de elucidar características do genoma do feijão-caupi como um todo, este trabalho integra marcadores moleculares com características fenotípicas em um mapa genético de ligação, analisando as informações dos projetos de sequenciamento que representam transcritos de vários genótipos, em diferentes estádios de desenvolvimento, órgãos, tecidos e condições de estresse. Trabalhos como este, são essenciais para futuras ações visando ao incremento da produção agrícola mundial do feijão-caupi, devendo ter também implicações de fundamental importância no melhoramento e na biotecnologia desta cultura, abrindo novas perspectivas para modificações genéticas tanto nos aspectos visuais, como cor e forma, como também nos metabólitos relacionados à qualidade nutricional, defesa contra fitopatógenos e estresses ambientais.

REVISÃO BIBLIOGRÁFICA

1. Genômica Vegetal: princípios e métodos

Até recentemente, a biologia molecular era utilizada para isolar e estudar um único ou poucos genes, bem como analisar os efeitos fenotípicos de interesse para agricultura, o que limitava bastante o entendimento das bases moleculares de genes e das características fenotípicas complexas resultantes da ação de muitos genes, como por exemplo, produtividade e resistência a doenças. O desenvolvimento de novas tecnologias têm permitido o sequenciamento completo dos nucleotídeos de um genoma e a análise da expressão de milhares de genes em plantas submetidas a diferentes condições ambientais ou sujeitas a estresses bióticos e abióticos, colocando nas mãos dos pesquisadores ferramentas que permitem uma nova visão dos mecanismos envolvidos desde a manutenção da estrutura e função ao nível celular até as intricadas interações de numerosos genes. Assim, as plantas – cujos modelos serviram de base para o desenvolvimento da genética – entraram também na era genômica (Arruda, 2004).

De forma geral, a genômica pode ser dividida em três grandes áreas: estrutural, funcional e comparativa. A genômica estrutural estuda a estrutura e organização dos genes, sequências regulatórias e regiões não codificantes. Seu principal alvo é a geração de sequências gênicas e suas respectivas localizações nos cromossomos. Suas abordagens seguem o sequenciamento em larga escala, o mapeamento físico a partir da construção de bibliotecas de BACs (Cromossomos Bacterianos Artificiais, do inglês *Bacterial Artificial Chromosomes*), mapeamento genético, identificação de marcadores moleculares e análise estrutural e modelagem de proteínas (Benko-Iseppon *et al*., 2012).

A genômica vegetal experimentou grandes avanços a partir do sequenciamento completo do genoma de *Arabidopsis thaliana*, publicado no ano 2000 (AGI, 2000). Inovações em tecnologias de sequenciamento do DNA para geração de sequências em larga escala levou a uma mudança de paradigma na genômica vegetal (Lister *et al*., 2009). Uma revisão sobre a evolução histórica desta tecnologia é apresentada por Hutchison (2007). Uma das técnicas de sequenciamento mais utilizadas envolve a fragmentação randômica de DNA (*Shotgun*), havendo várias tecnologias emergentes de sequenciamento que representam uma nova geração do *Shotgun* (Carvalho e Silva, 2010; Schlebuch e Illing, 2012).

O desenvolvimento de dados genômicos progrediu em várias espécies de angiospermas. A Tabela 1 lista as principais angiospermas, consideradas relevantes como culturas agrícolas de interesse

socioeconômico, com sequências depositadas em bancos públicos e privados. Um dos principais bancos de dados disponibilizado online é o NCBI (http://www.ncbi.nlm.nih.gov/), havendo outros particularmente direcionados para espécies vegetais, como no caso do Phytozome (www.phytozome.net), do Ensembl Plants (http://plants.ensembl.org/index.html) e do PlantGDB (www.plantgdb.org).

Alguns bancos de dados são específicos como o GDR (http://www.rosaceae.org/) para família Rosaceae que abriga a maçã, o morango e o damasco (Jung *et al*., 2008); o SOL (solgenomics.net) para família Solanaceae da qual fazem parte a batata e o tomate (Bombarely *et al*., 2011) e o LegumeIP (http://plantgrn.noble.org/LegumeIP/) plataforma para estudo de genes e evolução em leguminosas modelo (Li *et al*., 2011). Esse grande número de sequências tem gerado uma explosão de informações e inovação em diversas áreas de pesquisa, especialmente na fitossanidade e no melhoramento genético de plantas (Carrer *et al*., 2010).

Tabela 1. Genomas de plantas concluídos ou em fase final de sequenciamento, bem como o tamanho correspondente, ordenadas cronologicamente.

Planta	Genoma (Mb)	Referências	Progresso
Oryza sativa (arroz)	389	Yu *et al*. (2002)	Completo
Vitis vinifera (videira)	500	Velasco *et al*. (2007)	Completo
Medicago truncatula	500	MGSC (2007) Consórcio	Completo
Lotus japonicus (lótus)	470	Sato *et al*. (2008)	Completo
Sorghum bicolor (sorgo)	736	Paterson *et al*. (2009)	Completo
Cucumis sativus (pepino)	370	Huang *et al*. (2009)	Completo
Zea mays (milho)	2500	Wei *et al*. (2009)	Completo
Hordeum vulgare (cevada)	5000	Schulte *et al*. (2009)	Andamento
Vigna radiata (feijão-mungo)	515	Tangphatsornruang *et al*. (2009)	Andamento
Glycine max (soja)	1200	Schmutz *et al*. (2010)	Completo
Ricinus communis (mamona)	400	Chan *et al*. (2010)	Completo
Malus x *domestica* (maçã)	750	Velasco *et al*. (2010)	Completo
Theobroma cacao (cacau)	430	Argout *et al*. (2010)	Completo
Jatropha curcas (pinhão-manso)	400	Sato *et al*. (2011)	Completo
Solanum tuberosum (batata)	840	PGSC (2011) Consórcio	Andamento
Manihot esculenta (mandioca)	770	Prochnik *et al*. (2012)	Completo
Musa acuminata (banana)	600	D'Hont *et al*. (2012)	Andamento
Solanum lycorpesicum (tomate)	900	TGC (2012) Consórcio	Andamento
Cucumis melo (melão)	480	Garcia-Mas *et al*. (2012)	Completo

O conhecimento das sequências do genoma das plantas cultivadas, por si só, não é o objetivo final da pesquisa genômica. É importante identificar as implicações das características genômicas no funcionamento celular, fisiológico e molecular dos organismos. Como consequência, surgiu a 'Genômica Funcional' que tem como objetivo primordial determinar a função e questões espaciais e temporais da expressão dos diversos genes. Basicamente, a função de um gene é determinada quando são identificados os seus respectivos produtos gênicos, ou seja, seu RNA mensageiro (RNAm), bem como as proteínas e os metabólitos correspondentes. Portanto, a genômica funcional abrange estudos do transcriptoma, proteoma, metaboloma e de biologia dos sistemas. Tradicionalmente a transcriptômica compreende a caracterização e a quantificação do conjunto de transcritos (moléculas de RNAm) nas células. Assim, as ferramentas de genômica funcional buscam responder algumas questões básicas que incluem aspectos como: quando o gene é expresso, onde o produto está localizado, em quais interações o gene se envolve e em que fenótipo resulta quando ele sofre mutação. A genômica funcional aspira responder sistematicamente questões envolvendo muitos genes simultaneamente até todos os genes do genoma, ao contrário das abordagens convencionais que estudam um gene por vez (Steinmetz e Davi, 2004; Villas-Bôas e Gombert, 2006).

A análise dos produtos gênicos em plantas requer o uso de técnicas analíticas que gerem dados suficientes para serem combinados às informações já obtidas com o sequenciamento dos genomas. Os primeiros estudos do transcriptoma se basearam no isolamento do RNA total de diferentes tecidos e uso da técnica de *Northern blot* (hibridização DNA-RNA) para detecção dos transcritos. As limitações desta técnica foram superadas pelo uso da reação em cadeia da polimerase (PCR, do inglês *Polymerase Chain Reaction*) via transcriptase reversa quantitativa (qRT-PCR) (Santana, 2011). O método quantitativo de PCR em tempo real tem sido considerado importante devido à alta sensibilidade, especificidade, reprodutibilidade e amplo desempenho de quantificação dos genes de interesse (Udvardi *et al*., 2008). Este método tem como base a obtenção de sinal de fluorescência (fluoróforo) durante cada amplificação de PCR comum que, simultaneamente amplifica, quantifica e analisa a expressão gênica. O fluoróforo aplicado no método pode ser uma ou mais sondas ligadas aos *primers*, como observado no sistema *TaqMan*, ou moléculas de *SYBR Green* que se ligam entre a fita dupla de DNA e com a excitação da luz emitida pelo sistema ótico do termociclador, emitindo uma fluorescência verde (Gachon *et al*., 2004; Novais e Pires-Alves, 2004; Deepak *et al*., 2007).

Com a disponibilidade de sequências genômicas completas para muitas plantas, *chips* de DNA ou microarranjos (tradução do termo em inglês *microarrays*) de alta densidade foram criados para

analisar a expressão de milhares de genes simultaneamente, o que permitiu a geração de um número substancial de dados, propiciando investigações globais a respeito de atividades celulares e expressão diferencial de genes (Rensink e Buell, 2005; Gregory *et al*., 2008). Ao mesmo tempo em que as metodologias baseadas em *microarrays* oferecem um excelente potencial para análise de milhares de genes, estas metodologias trazem resultados mais robustos, quanto maior a quantidade de informação genética disponível para o uso dessa metodologia na espécie em questão (Carvalho, 2006). O feijão-caupi não tem ainda uma plataforma comercial de *microarrays* disponível, motivo pelo qual os estudos publicados utilizam a metodologia desenvolvida para soja (Das *et al*., 2010).

A geração de EST (Etiquetas de Sequências Expressas ou *Expressed Sequence Tags*) também se classifica como uma tecnologia de análise de expressão gênica em larga escala, sendo muito rentável em termos de quantidade e qualidade da informação gerada (Marques e Silva, 2004). Estas bibliotecas são desenvolvidas a partir do universo de RNAm isolados da amostra de interesse, utilizando a enzima transcriptase reversa, que gera um DNA complementar (cDNA) ao RNAm. Ao contrário de uma biblioteca genômica, que contém em princípio qualquer fragmento de DNA do organismo doador, a biblioteca de cDNA contém os genes expressos no tempo e local específicos. Esses seguimentos de cDNA são clonados, selecionados aleatoriamente e sequenciados, gerando sequências de aproximadamente 300-600 pb que são então identificadas através de análises *in silico* com ferramentas de Bioinformática (Adams *et al*., 1991; Malone *et al*., 2006).

No Brasil são várias as iniciativas de consórcios de pesquisadores nos projetos de EST como: (i) Projeto de Etiquetas de Sequências Expressas de Cana-de-Açúcar (SUCEST - *Sugarcane Expressed Sequence Tags*; http://watson.fapesp.br/sucest.htm); (ii) Projeto de Etiquetas de Sequências Expressas de Eucalipto (FORESTs - https://forests.esalq.usp.br); (iii) Projeto Genoma da Soja (*Soybean Genome Project*; GENOSOJA – http:// www.lge.ibi.unicamp.br/soja); (iv) Projeto Transcriptoma do Feijão-Caupi (*Cowpea Transcriptome Project*; NordEST – http://bioinfo03.ibi.unicamp.br/vigna/); (v) Projeto de Sequências Expressas de Citros (CitEST- http://biotecnologia.centrodecitricultura.br) e (vi) Projeto Genoma Brasileiro Café (CoffeaEST - http://www2.iict.pt/?idc=15&idi=18340). Esses programas vêm sendo financiados pelas agências federais (CNPq, Capes e Finep) e estaduais (FAPESP, FAPEMIG, FACEPE e outras) junto com as universidades e empresas públicas (geralmente federais), vários deles em parcerias com a Embrapa (Empresa Brasileira de Pesquisa Agropecuária) e o Instituto Agronômico de Campinas (Vettore *et al*., 2001; Vieira *et al*. 2006; Targon *et al*., 2007; Benko-Iseppon *et al*., 2012).

Estas iniciativas tiveram grande impacto científico internacional e abriram caminhos para aplicações em programas de melhoramento, visando atender às necessidades de diferentes regiões do país.

Estima-se que até 2011 foram sequenciados mais 21 milhões de fragmentos de ESTs em plantas (Blair *et al*., 2011a). Coleções de ESTs são importantes para estudo da estrutura dos genes e inferência da sua função, refletindo o nível e complexidade de expressão gênica e de vias metabólicas a que estão sujeitos os processos fisiológicos, desenvolvimento e respostas a estímulos. Este método tem sido empregado para identificar os genes que são expressos em vários tecidos, tipos celulares ou estágio de desenvolvimento, genes expressos em interação planta-microrganismo patogênicos e não patogênicos, como também sob situações de estresse abiótico (Guo *et al*., 2008; Blair *et al*., 2011b; Yin *et al*., 2011; Oblessuc *et* al., 2012; Pestana-Calsa *et* al., 2012; Soares-Cavalcanti *et al*., 2012a; Wanderley-Nogueira *et al*., 2012). Além das ESTs, a SAGE (Análise Serial da Expressão Gênica, do inglês *Serial Analysis of Gene Expression*) e suas variações (microSAGE, miniSAGE, LongSAGE e SuperSAGE) envolvem outra tecnologia que tem demonstrado grande eficiência e facilidade para o estudo de expressão diferencial de genes. Nas técnicas de SAGE e suas variações as etiquetas de cDNA apresentam de 9-21 pares de bases (*tag*) obtidas da extremidade 3' de cada molécula de RNAm. As *tags* são extraídas por uma série de ligações com adaptadores e digestões com enzimas de restrição. Em seguida, as *tags* são ligadas entre si, formando uma longa molécula, denominada concatâmeros, que são clonados e sequenciados.A análise do sequenciamento é feita com auxílio de um software e a contagem de cada *tag* representa o nível de expressão do transcrito (Dinel *et al*., 2005).

Conforme revisado por Kido *et al*. (2012a), a adequação da técnica SuperSAGE (que gera *tags* com 26 bp) em combinação com as tecnologias de sequenciamento de segunda geração (*Next Generation Sequencing*, NGS; Matsumura *et al*., 2012), incluindo plataformas como Roche® 454 (Molina *et al*., 2008; Ito-Inaba *et al*., 2012), Illumina (Solexa®) (Yamaguchi *et al*., 2010; Kido *et al*., 2010, 2011, 2012b, 2012c) e SOLid (Applied Biosystems®) (Matsumura *et al*., 2012) é denominada HT-SuperSAGE (*High Throughput*) ou deepSuperSAGE ou ST-DGE (SuperTAG *Digital Gene Expression Technology*, http://www.genxpro.info/science_and_technologies/) fornecendo uma abordagem altamente sensível, capaz de gerar mais dados de sequências com três a quatro ordens de magnitude comparativamente às metodologias derivadas de SAGE que dependem de concatenação e com custos consideravelmente mais baixos. A associação da abordagem SuperSAGE com estes métodos proporciona uma amostra maior do transcriptoma (milhões de etiquetas), aumentando a sensibilidade e reduzindo os custos e tempo de produção (Matsumura *et al*., 2011). Portanto, a técnica HT-SuperSAGE

tem sido usada com sucesso para estudar as respostas de muitas plantas, incluindo gramíneas (como arroz e cana de açúcar) e leguminosas sob estresses bióticos e abióticos (Molina *et al*., 2008; Kido *et al*., 2010; Kido *et al*., 2011).

Num artigo de referência, Molina *et al*. (2008) utilizaram o método SuperSAGE para acessar o perfil de expressão de grão de bico (*Cicer arietinum* L.) submetido ao déficit hídrico, envolvendo a criação de um perfil abrangente da transcrição para esta espécie de leguminosa. Mais tarde, o transcriptoma das raízes e nódulos de plantas de grão de bico sensíveis e tolerantes à salinidade através de deepSuperSAGE foi acessado por Molina *et al*. (2011), revelando candidatos adicionais especificamente induzidos por este tipo de estresse em ambos os tipos de tecidos. A mesma técnica foi aplicada ao genoma da soja (Benko-Iseppon *et al*., 2012) associada a análises envolvendo estresse hídrico e resistência à ferrugem asiática. A quantificação da expressão dos genes, avaliada por meio do número de vezes que uma determinada *tag* é encontrada na planta sob um determinado estresse biótico ou abiótico a ser investigado comparado ao número de vezes em que é encontrado na mesma planta sem estresse (controle). Assim, se um número significativamente maior de *tags* for observado nas plantas estressadas em comparação ao número obtido nas plantas controle, é dito que o gene por ela representado tem expressão aumentada quando a planta sofre o estímulo do estresse. Se o número de *tags* for significativamente menor na planta estressada o que no encontrado na planta controle, é dito que o gene por ela representado tem expressão diminuída no estresse. Esta metodologia foi descrita em 1995 e já está bem estabelecida, sendo amplamente utilizada em diferentes projetos com uso da técnica de SAGE com sequenciamento Sanger (Velculescu *et al*., 1995; 2000), bem como com sequenciamento de segunda geração (HT-SuperSAGE; Molina *et al*., 2011; Kido *et al*., 2012b, 2012c; Soares-Cavalcanti *et al*., 2012b), tendo esta última sido aplicada inclusive no projeto genoma do feijão-caupi (rede NordEST) na geração de bibliotecas relacionadas a estresses bióticos (doenças virais, Potyvírus e Mosaico Severo) e abióticos (seca e salinidade).

A proteômica também estuda a expressão gênica, mas em nível de tradução, possibilitando avaliações qualitativas e quantitativas de proteínas que atuam no metabolismo celular, o estudo da expressão diferencial das proteínas, bem como de modificações pós-traducionais e associadas à interação proteína-proteína (Chen e Harmon, 2006; Sider e Zaros, 2008). É uma ferramenta eficaz para análise do estado fisiológico da planta, de processos específicos e também avaliação das respostas a fatores bióticos e abióticos, incluindo alterações no genoma (Rossignol *et al*., 2006).

Nesses estudos, utilizam-se técnicas de eletroforese bidimensional em gel de poliacrilamida (2D PAGE) para separar e comparar as proteínas presentes em diferentes tecidos das plantas, aplicando-se em seguida a técnica de espectrometria de massa (MS) e análises com o auxílio de ferramentas de bioinformática para interpretação dos dados (Rocha *et al*.,2005; Isaaq e Veenstra, 2008).

A caracterização do proteoma com a identificação do conjunto total de proteínas ou das proteínas diferencialmente expressas entre duas amostras com características fenotípicas contrastantes é um procedimento adotado na proteômica vegetal. A grande maioria dos estudos de proteômica de plantas é voltada para os perfis proteicos de vários órgãos, tecidos, células e organelas das principais espécies cultivadas. Estes estudos têm implicações diretas em vários campos da biologia e da biotecnologia. Dentre suas vertentes comprova-se a descoberta de vias metabólicas nas diversas etapas celulares, favorecendo o conhecimento na biologia celular e da bioquímica, viabilizando a identificação de moléculas bioativas que podem ser aplicadas no desenvolvimento de novos medicamentos (Rocha *et al*., 2005).

As análises de proteômica em leguminosas têm sido utilizadas em estudos de interação planta-microrganismo (Larrainzar *et al*., 2007; Castillejo *et al*., 2009; Cooper *et al*., 2011), desenvolvimento das sementes (Agrawal *et al*., 2008) e proteínas com potencial alergênico (Xu *et al*., 2007). Análises deste tipo envolvendo o feijão-caupi são ainda escassas. Nogueira *et al*. (2007) utilizaram células embriogênicas em suspensão para obter o perfil proteico em feijão-caupi. Os autores identificaram várias proteínas relacionadas com o crescimento, divisão celular, atividade hormonal e proteínas relacionadas à patogênese (*Pathogenesis Related* ou PR) que são produzidas em resposta a estresses físicos, químicos ou biológicos. Outros autores avaliaram o perfil proteico relacionado à toxicidade do manganês, identificando a interferência deste elemento em vários processos moleculares como estresse oxidativo e metabolismo enzimático (Nogueira *et al*., 2007; Führs *et al*., 2009).

A área de metabolômica surgiu recentemente como ciência que estuda a caracterização de todos os metabólicos intracelulares e extracelulares em diferentes condições, visando à descoberta de biomarcadores ou a compreensão das reações envolvendo tais metabólitos. Para tal, devem-se encontrar compostos que estão presentes em apenas uma das condições avaliadas ou compostos que têm sua quantidade variada significativamente de uma condição para outra. O nível de cada metabólito vai depender do estado fisiológico, de desenvolvimento, e/ou patológico de uma célula, tecido ou organismo (Villa-Bôas *et al*., 2005; Pedroso *et al*., 2009). A Figura 1 ilustra, de forma resumida, os possíveis

pontos de regulação em nível molecular do funcionamento celular de acordo com Villas-Bôas e Gombert (2006).

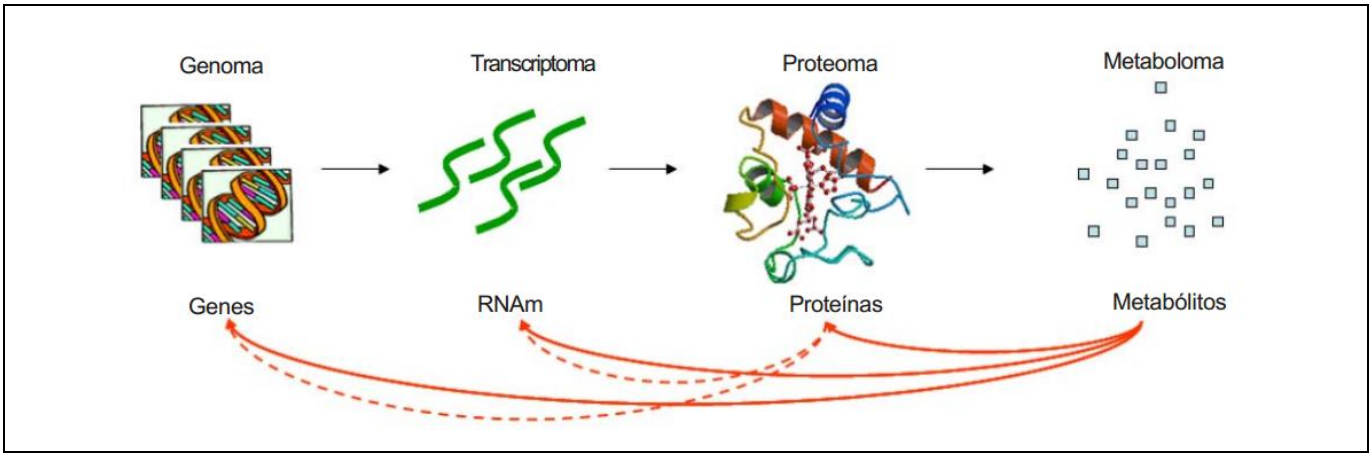

Figura 1. Funcionamento celular em nível molecular. A informação codificada no DNA é transcrita em uma molécula de RNA mensageiro (RNAm) que é traduzida em uma molécula de proteína que, quando não exerce um papel estrutural, será responsável pela transformação de pequenas moléculas químicas em metabólitos. Os metabólitos, por sua vez, regulam a atividade das proteínas, o processo de transcrição e de tradução, além de servirem de substrato para modificação e síntese de proteínas, lipídios, bem como a síntese de DNA e RNA. Assim os metabólitos são os produtos finais do metabolismo celular (Fonte: Villas-Bôas e Gombert, 2006).

A genômica é conhecida como a ciência que se dedica ao sequenciamento dos nucleotídeos, mapeamento e análise de genomas, acreditando-se que formará a base do que pode ser denominado o agronegócio do futuro, sendo associada não somente ao mercado de alimentos e derivados, mas também à expansão para o mercado de biomoléculas de alto valor agregado que serão utilizadas como diagnósticos, fármacos, vacinas, polímeros, terapia gênica e proteica, bioensaios e sistemas de alta produção em nível bioquímico e biofísico (Rech Filho, 2004; Furlan *et al*., 2007). O ponto chave é que não se trata apenas de uma técnica que vai ajudar a identificar a função do gene com potencial biotecnológico, mas considerando uma abordagem integrada como foco. As informações obtidas com as "ômicas" podem ser complementadas com a geração e caracterização de mutantes e o mapeamento físico e genético, bem como com o uso de técnicas de transgenia (Tyagi *et al*., 2006).

2. Mapeamento Físico e Citogenética em *Vigna*

A informação genética do feijão-caupi está contida em 11 pares de cromossomos (Figura 2), os quais apresentam entre 1,25 e 2,1 µm e podem ser distinguidos por diferenças no tamanho e na quantidade de heterocromatina (Bortoleti *et al*., 2012). O seu genoma diploide apresenta 620 Mb e estima-se que aproximadamente 26% das sequências sejam ricas em genes (*gene rich-space*) (Timko *et al*., 2008).

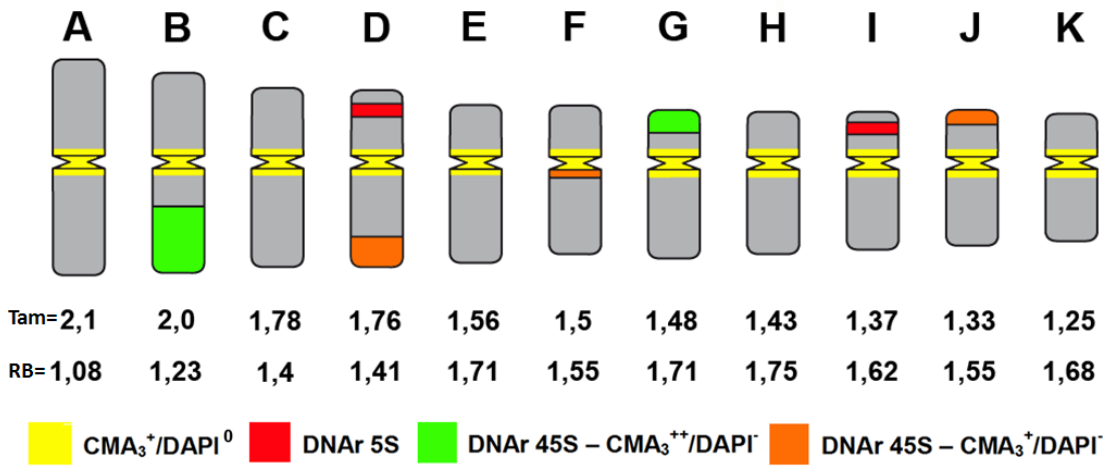

Figura 2. Ideograma médio representando os cromossomos mitóticos de feijão-caupi (*Vigna unguiculata*) com a localização do centrômero, distribuição de heterocromatina e DNA ribossomal 5S e 45S, $CMA_3^{++}/DAPI^-$. Abreviações: Tam=Tamanho (em micrômetros) e RB=Razão entre os braços. Fonte: Bortoleti *et al*. (2012).

A citogenética molecular usa ferramentas poderosas para análises da estrutura, organização e evolução genômica. Neste contexto, a técnica de FISH (*Fluorescent In Situ Hybridization*; Hibridização *In Situ* Fluorescente) se destaca por permitir a localização de regiões cromossômicas específicas nas mais variadas espécies de plantas silvestres e cultivadas, bem como o estabelecimento de agrupamentos de ligação genética com os cromossomos (ligação de mapa genético e físico). Também permite que regiões associadas a características de interesse possam ser rastreadas em programas de melhoramento, além da identificação de alterações cromossômicas em cruzamentos interespecíficos. O processo consiste em desnaturar e hibridizar com uma sonda adequada (DNA ou RNA conhecido e previamente

marcado) os fragmentos do ácido nucleico alvo, possibilitando a localização *in situ* de sequências de ácidos nucleicos nos cromossomos, citoplasma, organelas ou tecidos (Guerra, 2004; Proite *et al*., 2006).

Em leguminosas, a crescente aplicação de técnicas de bandeamento cromossômico, notadamente as de bandeamento C e o emprego de fluorocromos base específicos (CMA_3 e DAPI), bem como FISH com sondas de DNA ribossomal (DNAr) e de elementos transponíveis, tem permitido não só uma melhor caracterização da diversidade cariotípica, como também o entendimento das relações evolutivas existentes entre diferentes táxons, a construção de mapas cromossômicos e a integração entre mapas genéticos e cromossômicos (Galasso *et al*., 1995; Galasso *et al.*, 1997; Guerra *et al*., 1996; Ohmido *et al.*, 2007; Fonsêca *et al.*, 2010; Findley *et al*., 2011; Belarmino *et al*., 2012; Bortoleti *et al*., 2012).

As regiões de DNA repetitivo são os principais alvos das pesquisas citogenéticas em leguminosas visto que estes elementos são responsáveis por variações cariotípicas e desempenham grande papel evolutivo na organização genômica, cujas funções biológicas exercidas têm sido extensivamente discutidas. Inseridos no grupo de sequências repetitivas curtas e longas, encontram-se os elementos transponíveis, que correspondem a elementos móveis, ou seja, que têm a capacidade de se mover no genoma (Jurka *et al*., 2005). Em plantas, os elementos transponíveis estão separados em duas classes de acordo com a forma de transposição. Elementos de classe I ou retrotransposons são aqueles em que a transposição é realizada por intermédio de uma molécula de RNA, via mecanismo do tipo "copia e cola", o qual sofre transcrição reversa para sua reinserção no genoma. Os elementos de classe II ou transposons, por sua vez, codificam uma transposase, movimentando-se pelo genoma mediante um DNA intermediário, via mecanismo do tipo "corta e cola" (Todorovska, 2007; Du *et al*., 2010a). Dada a grande quantidade de elementos transponíveis em diferentes leguminosas, a identificação e caracterização destes segmentos têm sido de grande importância para uma melhor compreensão de seu papel na organização e evolução do genoma (Nielen *et al*., 2010; Xu *et al*., 2010; Civáň *et al*., 2011; Takahashi *et al*., 2012).

Os projetos de sequenciamento de genomas vegetais tem proporcionado um aumento das informações disponíveis sobre elementos de transposição, possibilitando o desenvolvimento de novas abordagens para seus estudos, incluindo desenvolvimento de sondas para FISH e de *primers* para amplificação e análise do número de cópias e de prováveis regiões de inserção no genoma, bem como análises *in silico* para inferir sobre o papel desses elementos na organização do genoma a partir da análise da abundância, número de cópias e associação a outras sequências.

Em feijão-caupi os trabalhos disponíveis referem-se ao uso da FISH para identificação de retrotransposon do tipo Ty1-*copia*-like e Ty3-*gypsy* (Galasso *et al.* 1997; Bortoleti, 2010), com sondas consideradas universais e utilizadas em várias espécies de plantas (Fregonezi *et al*., 2007; Jiang *et al*., 2010). No caso da soja, existe um banco de dados com as sequências dos elementos transponíveis identificados a partir do sequenciamento completo do genoma (Du *et al*., 2010b). A publicação da plataforma de bioinformática desta cultura permitiu a mineração e integração de conhecimentos entre diferentes leguminosas, incluindo o feijão-caupi. A identificação de diferentes classes de elementos transponíveis no genoma de feijão-caupi através de ferramentas computacionais poderá auxiliar diversas pesquisas na área biológica e molecular, antecipando os estudos experimentais de alterações cromossômicas estruturais (rearranjos) e funcionais (alterações da expressão gênica).

Além da identificação de regiões repetitivas, o mapeamento físico serve de base para o isolamento de genes de interesse agronômico, técnica também conhecida como clonagem posicional, sendo necessária a construção de uma biblioteca que tenha grandes quantidades de DNA genômico, como BACs (Moretzsohn, 2006). Recentemente, várias bibliotecas BACs foram construídas em leguminosas cultivadas incluindo o feijão-mungo, feijão-caupi, grão-de-bico (*Cicer arietinum*), ervilha (*Pisum sativum*) e o feijão-comum (*Phaseolus vulgaris*) (Yu, 2012). Embora tenha sido disponibilizada uma quantidade considerável de informação para o genoma de feijão-caupi, marcadores não foram gerados para hibridizar com as bibliotecas de BACs nos cromossomos. Assim, uma das metas do projeto NordEST é a confecção de um mapa físico baseados em BACs com sondas específicas para genes de resistências a doenças virais e peptídeos antimicrobianos. Atualmente as atividades de pesquisas envolvem a hibridização de BACs de feijão-comum em feijão-caupi, a fim de colaborar para a análise da sintenia existente entre as duas espécies (Vasconcelos, 2011).

3. Mapeamento genético e marcadores moleculares em leguminosas

Marcador molecular é definido por Ferreira e Grattapaglia (1998) como sendo todo e qualquer fenótipo molecular oriundo de um gene expresso (isoenzimas) ou de um segmento específico de DNA que pode corresponder a uma região expressa ou não do genoma. Atualmente, diversos marcadores moleculares com características específicas estão disponíveis, sendo que os mais modernos em geral são

desenvolvidos para espécies-modelo ou de importância econômica (Goulart *et al*., 2011). Esse 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*), DAF (Impressão Genômica da Amplificação do DNA, do inglês *DNA Amplification Fingerprinting*), AFLP (Polimorfismo de Comprimento de Fragmentos Amplificados, do inglês *Amplified Fragment Length Polymorphism*), SSR (Sequência Simples Repetida, do inglês *Simple Sequence Repeat*), DarTs (Tecnologia de Arranjos de Diversidade, do inglês *Diversity Arrays Technology*) e CAPS (Polimorfismo de Sequência Amplificada e Clivada, do inglês *Cleaved Amplified Polymorphic Sequence*) (Semagn *et al*., 2006; Agarwal *et al*., 2008; Petroli *et al*., 2012).

O uso do mapeamento genético para estudos comparativos ou de sintenia contribui para o entendimento sobre a evolução dos genomas. A sintenia é caracterizada por um grau de similaridade ou conservação entre regiões de genomas distintos que não poderia ser explicado como uma simples casualidade. Para observar a sintenia é necessária a comparação das estruturas genômicas de diferentes espécies com uso de ferramentas moleculares visando identificar a ocorrência de conservação na ordem e no conteúdo dos genes, ou grupos gênicos. Essa característica também é denominada colinearidade, sendo informativa para estudos comparativos de função, ação e regulação gênica entre diferentes genomas (Carneiro e Viera, 2002; Zimmer *et al*., 2003; Catanho *et al*., 2007).

Diversos estudos comparativos têm revelado um alto grau de sintenia e colinearidade de regiões genômicas e gênicas entre espécies de leguminosas (Choi *et al*., 2004; Hougaard *et al*., 2008; Bertioli *et al*., 2009; Gaur *et al*., 2012) ou por vezes mesmo entre espécies distantemente relacionadas (Benko-Iseppon et al., 2003).

Em feijão-caupi os estudos de mapeamento comparativo são recentes e escassos. Muchero *et al*. (2009a) e Lucas *et al.* (2011) construíram mapas genéticos em feijão-caupi com marcadores SNPs (Polimorfismo de Base Única, do inglês *Single Nucleotide Polimorphism*) e na análise de sintenia com soja e com *Medicago truncatula*, observando elevados níveis de conservação genômica ou macrossintenia em todos os grupos de ligação. Com base nesses estudos, pode-se esperar que as estratégias de transferência de conhecimento e tecnologia em leguminosas sejam úteis na identificação dos mecanismos de evolução de genes de tolerância a estresses bióticos e abióticos e de genes com função desconhecida, informações que podem ser aplicadas em programas de melhoramento convencional e em no desenvolvimento de tecnologias de transformação genética.

Além de estudos comparativos, os mapas genéticos desenvolvidos para espécies do gênero *Vigna* podem ser classificados em: 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 (Agbicodo *et al*., 2010; Rodrigues *et al*., 2012).

Menéndez *et al*. (1997) construíram o primeiro mapa genético com um cruzamento intraespecífico de feijão-caupi. 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. Ouédraogo *et al*. (2002) utilizando a população de mapeamento empregadas 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 duas raças de *Striga gesnerioides*, nematoide das galhas, viroses e fungos. Vale salientar, no entanto, que a rede NordEST é a principal iniciativa para construção de um mapa de referência associados a marcadores moleculares a partir de um cruzamento envolvendo uma cultivar brasileira de feijão-caupi e com ênfase na resistência a viroses (CPSMV e CABMV), que figuram entre os agentes patogênicos mais impactantes na cultura do feijão-caupi em regiões tropicais e secas.

Uma primeira versão do sequenciamento do genoma do feijão-caupi foi divulgada em 2008 pelo grupo de pesquisa dos Estados Unidos. Para reduzir os custos do sequenciamento do genoma completo, os autores sequenciaram as regiões ricas em genes, selecionadas a partir do padrão de metilação (*Cowpea GeneSpace*; Timko *et al*., 2008). A análise das sequências geradas no projeto propiciou a identificação de uma grande coleção de polimorfismos genéticos, na sua maioria SNPs, que culminou com a elaboração de um mapa de 928 SNPs perfazendo um comprimento total de 680 cM em 11 grupos de ligação, com uma distância de 0,73 cM entre os marcadores (Muchero *et al*., 2009a), dados que atualmente estão sendo úteis para identificação de QTLs. Estudos adicionais focaram no mapeamento de QTLs para característica de resistência a doenças e a pragas. Muchero *et al*. (2009b) encontraram 12 locos QTLs relacionados com a tolerância à seca e à maturidade de plântulas de feijão-caupi. Por sua vez, Muchero *et al*. (2010) identificaram três locos QTLs associados à resistência às pragas *Thrips tabaci* e *Frankliniela schultzei*. Um trabalho mais recente do mesmo grupo (Muchero *et al*., 2011) reportou o mapeamento de oito QTLs (*Mac1* a *Mac8*) distribuídos em quatro grupos de ligação associados à resistência a *Macrophomina phaseolina*.

Apesar do sucesso obtido nos estudos de mapeamento de QTLs em feijão-caupi, ainda não há marcadores de DNA sendo utilizados em programas de melhoramento genético no Brasil. Com a saturação do mapa genético desenvolvido no âmbito do NordEST espera-se aumentar a chance de eficiência na localização de QTLs e de genes candidatos.

Marcadores SSR também têm sido empregados com sucesso na saturação de mapas genéticos no gênero *Vigna*. A maior vantagem dos marcadores microssatélites é que eles são facilmente transferíveis de um mapa para outro, sendo muito úteis também no alinhamento de mapas. Chaitieng *et al*. (2006) desenvolveram o primeiro mapa de ligação para *V. mungo* com 145 marcadores, sendo 61 SSR, 56 RFLPs, 27 AFLPs e 1 marcador morfológico. Os marcadores formaram 11 grupos de ligação com uma distância média entre os marcadores de 5,7 cM, totalizando 768 cM. O mapa de ligação construído, quando comparado com o mapa de *V. angularis* (Han *et al*., 2005), mostra que a conservação na ligação entre os locos e a ordem dos marcadores SSR é alta.

Onofre *et al*. (2008) avaliaram 20 microssatélites desenvolvidos para espécies do gênero *Vigna* por diferentes grupos de pesquisa, analisando a transferibilidade dos mesmos para o feijão-caupi concluindo que devido à conservação dos sítios que flanqueiam os microssatélites, é possível amplificar *primers* heterólogos nas diferentes espécies do gênero. Dos iniciadores analisados 19 foram consistentemente polimórficos e transferíveis, sendo então recomendados para o desenvolvimento de mapas de ligação em feijão-caupi e estudo de sintenia.

Os bancos de dados de EST e os transcritos ou *tags* obtidos pela técnica de HT-SuperSAGE podem fornecer um recurso útil para desenvolvimento de marcadores. Existem várias vantagens em realizar estudos genéticos com marcadores EST-PCR ou outros baseados em cDNA em relação aos que utilizam os *primers* aleatórios. Eles marcam genes expressos e, portanto são particularmente úteis para o mapeamento fino e de QTL. Se um marcador EST-PCR é encontrado ligado a um QTL, é possível que o gene, a partir do qual o marcador EST-PCR foi derivado, controle a característica em questão. Pelo fato de serem originados de regiões codificadoras, que são mais conservadas entre populações e espécies do que as não codificadoras, os marcadores EST-PCR são úteis para o estudo de mapeamento comparativo (Rowland *et al*., 2003).

Os EST-PCR foram derivados para várias espécies vegetais e têm sido amplamente usados para diversas aplicações (Varshney *et al*., 2005; Miura *et al*., 2007; Proite *et al*., 2007). Por exemplo, 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.

Gupta e Gopalakrishma (2010) desenvolveram e avaliaram 102 marcadores SSR para feijão-caupi a partir de sequências expressas disponibilizadas no banco de dados do NCBI. Os marcadores apresentaram alta taxa de transferibilidade (88%) para outras espécies do gênero *Vigna*. Os autores consideram que estes marcadores são particularmente úteis no mapeamento genético e em estudos de genômica comparativa.

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).

A atual disponibilidade de técnicas de marcadores moleculares, as quais fornecem um elevado número de polimorfismos, permitirá grandes avanços no mapeamento genético de feijão-caupi nos próximos anos em relação a vários fatores determinantes na produção.

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 maturidade e precocidade. Os avanços na área de transformação genética apontam 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. No entanto, é de suma importância para a integração das informações geradas nas mais variadas áreas de pesquisas para melhorar a produção do feijão-caupi (Diouf, 2011).

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CAPÍTULO I
Artigo em Fase de Conclusão –
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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 12.5 million ha, with an annual production of over than 4.5 million tons (Singh *et al.* 2005). The largest takes place in Nigeria and Niger. Particulary in developing countries, cowpea has become a socio-economically relevant crop (Carvalho *et al*. 2012). The majority of cowpea production takes place in Nigeria and Niger. Other countries like Brazil, Haiti, India, Myanmar, Sri Lanka, Australia, the United States of America, and Bosnia and Herzegovina have also been recognized for significant production (Iyanda, 2013).

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. Material 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 89 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-808, 816 and 845 (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, CA and CT repeat motifs in their sequences. 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/OPH05/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.

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Table 1. Primer sequences, repeats, annealing temperature, 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

CAPÍTULO II
Artigo em Fase de Conclusão –
A ser Submetido à Revista Molecular Breeding

A LINKAGE MAP FOR COWPEA [*Vigna unguiculata* (L.) WALP.] AMONG A BRAZILIAN AND AN AFRICAN PARENTAL ACCESSION

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Abstract

Genetic mapping is 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 3.5 and a recombination frequency of 0.35. The current map includes 237 loci, mapped into twelve linkage groups (LGs), one more than the haploid chromosome number of cowpea. These were 98 DAF (DNA Amplification Fingerprinting) markers, 99 SSR (Simple Sequence Repeats) markers, 22 ISSR (Inter Simple Sequence Repeat) and 18 AFLP (Amplified Fragment Length Polymorphism) markers. The overall map length was 2,121.3 cM, with an average distance of markers of 9.3 cM. The length of each linkage group ranged from 69.7 (LG12) to 295 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 for comparative 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.

Keywords: cowpea, genetic map, molecular makers, breeding program.

1. Introduction

Genetic mapping associated with molecular markers brings valuable information for both, biological and agronomical 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 (as drought and salinity), plant architecture redesign and improvement of 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 Southern 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), AFLP (Amplified Fragment Length Polymorphism) and SSR (Simple Sequence Repeats) (Tan et al. 2012). According to Muchero et al. (2009a) access to most of the genes in cowpea can be gained through cDNA sequences, which represent expressed genes ESTs (Expressed Sequence Tags) sequecing 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). Additionaly, Xu et al. (2010) identified 1,010 SSR markers (410 eSSR and 600 GSS-SSR) from unigene sequences generated at HarvEST (University of California-Riverside) 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-2687
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 amplifications reactions were 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 Garcia 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 3.5 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 104 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 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, 68 polymorphic primer pairs were used in the RIL population, resulting in 71 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 27 primers pairs 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 parental accessions. 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-MB8, 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
SSR	220 markers	107 markers	110 bands	99 loci
DAF	105 markers	82 markers	104 bands	98 loci
ISSR	30 markers	24 markers	35 bands	22 loci
AFLP	64 markers	17 markers	59 bands	18 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			319 loci	237 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%)	68	71	66
Li et al. 2001	*V. unguiculata*	11	4 (36.36 %)	3	3	2
Han et al. 2005	*V. angularis*	33 (26 transferable)	10 (38.46%)	7	7	7
Gwag et al. 2006	*V. radiata*	1 (1 transferable)	1 (100%)	1	1	-
Garcia et al. 2011	*Phaseolus vulgaris*	38 (28 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%)	107	110	99

Construction of cowpea linkage map

Among the 319 loci used for preliminary mapping in the cowpea population (104 DAF, 59 AFLPs, 110 SSRs, 35 ISSR, 10 LEG/CAPS, and one phenotypic marker associated to Cowpea severe mosaic virus, CPSMV), 237 mapped to 12 linkage groups (LGs). Total map length was 2,121.3 cM, providing an average marker density of 1 marker per 9.3 cM. In our map, market densities varied significantly, due to pronounced clustering in some regions (especially in the LGs 2 and 5), as observed in Figure 1 and Table 4, where map features are presented. The length of LGs varied from 69.7 cM (LG12) to 295 cM (LG1). The number of marker loci per LG ranged from 6 to 34. LG1 and LG2 included most markers with an average distance of 8.7 cM and 6.6 cM, respectively.

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	295.0	34	8	03	03	20	8.7
LG2	225.4	34	21	00	04	09	6.6
LG3	222.3	29	09	01	01	18	7.7
LG4	270.5	20	09	03	02	06	13.5
LG5	135.9	20	12	03	01	04	6.8
LG6	187.6	18	07	03	01	07	10.4
LG7	176.8	19	06	01	03	09	9.3
LG8	165.9	18	04	00	00	14	9.2
LG9	172.5	17	08	01	04	04	10.1
LG10	101.0	12	07	01	02	02	8.4
LG11	98.7	10	04	01	00	05	9.9
LG12	69.7	06	03	01	01	01	11.6
Total	2,121.3	237	98	18	22	99	9.3

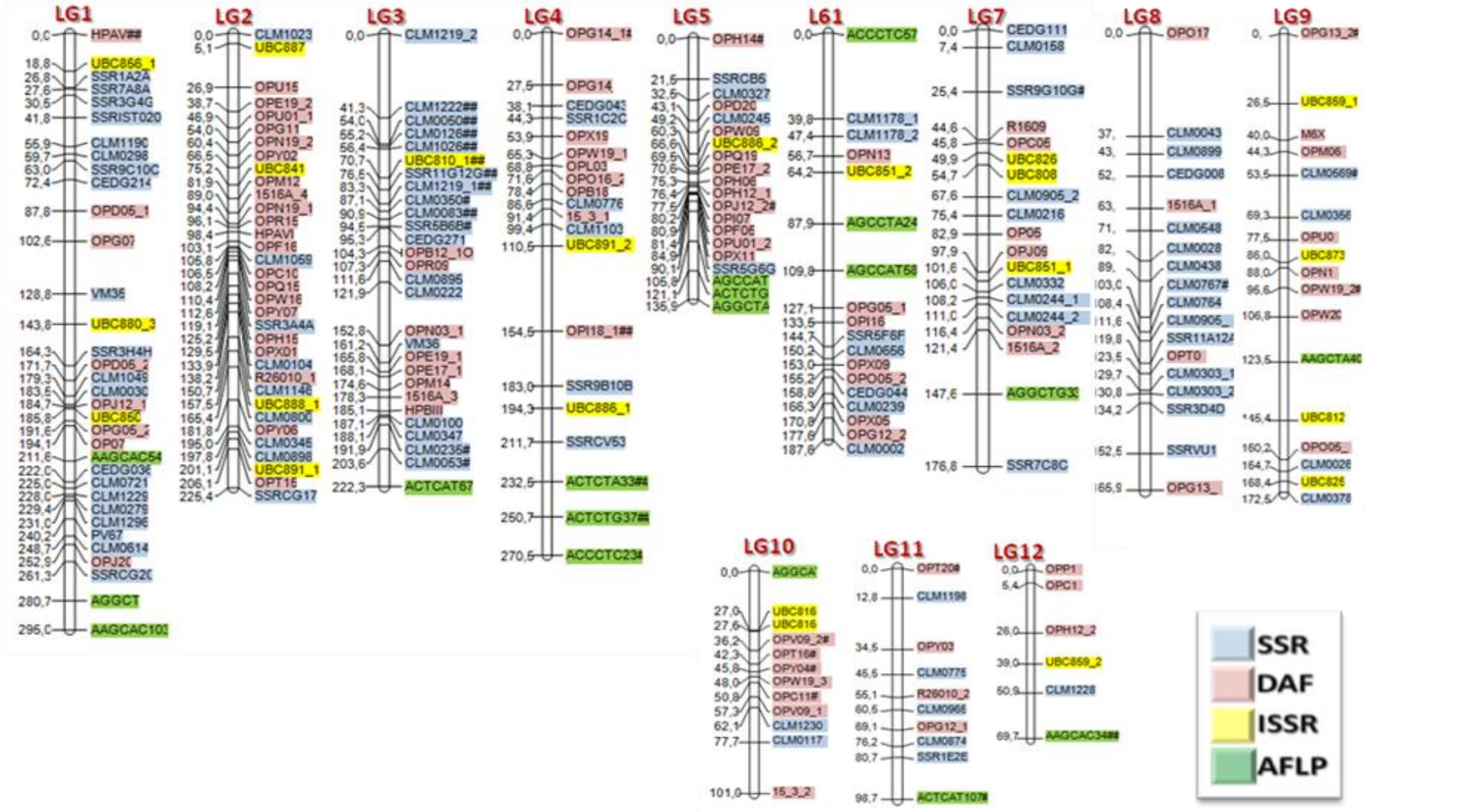

Figure 1. Genetic linkage map of cowpea (*Vigna unguiculata*) intraspecific cross based on a recombinant inbred population derived from a cross between BR14-Mulato and IT85F-2687. Cumulative recombination distances (in cM) are shown on the left and marker loci are shown on the right side of the linkage 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 and also their regular segregation and incorporation in the genetic map. In this study 98 out of 105 DAF loci (average almost one: 0.93) mapped across parental accessions. Menéndez et al. (1997) screened 332 RAPD primers on cowpea parental accessions (IT84S-2049 e 524B), from which only 133 loci mapped. So, they had about half of their proportion (133/332: 0.40). Gupta et al. (2008) used 360 random primers to screen polymorphisms across *V. mungo* parental accessions. They achieved 86 mapped loci, in a much smaller proportion than Menéndez et al. (1997), i.e., less than one loci for every four RAPD primers tested across parental lines (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 these 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.

In the generation of a wide cross map for chickpea (Winter et al., 2000), DAF were also the most informative and successful markers as compared with RAPD, that produced the most pronounced segregation distortion, while DAF presented the less pronounced distortion, with $\chi 2$ values less than ten (Winter et al., 2000).

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 (*V. unguiculata* ssp. *sesquipedalis*), 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 than fluorescent detection.

The majority of the polymorphic SSR markers fit the expected 1:1 segregation. However, segregation of 20 markers (8.4% of total) significantly deviated from this ratio ($P \leq 0.05$). The AFLP markers revealed high level segregation distortion (33.3%) when compared with the 13.6% for ISSR, 11.1% observed for DAF markers and 9.0% for SSR markers. Besides LEG/CAPS markers had no marker positioned on the map, AFLP showed lower number of markers allocated to twelve individual cowpea linkage groups.

The preliminary linkage map generated in the present study identified 12 linkage groups, one more than the haploid chromosome number of cowpea ($n = 11$). To reduce the number of the linkage groups, additional markers are needed for a better covered, high-density map. A significant amount of markers (82) 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 and in the telomeric region for LG1, LG3 and LG8. 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 (9.3 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.

Three other approaches have included QTL mapping directly linked to disease resistance, insect pest and leaf morphology. 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. Third, Pottorff et al. (2012) analysed and mapped QTL using an RIL population to identify genes which control leaf morphology.

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, a segregation analysis of the most valuable morphological features in the lines mapped and 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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CAPÍTULO III
Artigo em Fase de Conclusão –
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In silico analysis of class II transposable elements in cowpea as compared with actual soybean knowledge

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Abstract

The completion of plant genome sequences has enabled unprecedented systematic analysis of coding and non-coding DNA fractions. Transposable elements (TEs) are important genome components being still poorly investigated in cowpea. The recent publication of the cowpea genespace sequences opened the possibility for the evaluation of its transposon content. Using known TE from soybean as seed sequences we analyzed 153,476 sequences of the cowpea genespace database and found 1,612 TE elements belonging to six of the previously recognized superfamilies of class II elements: *Mutator* (736 sequences), *PIF/Harbinger* (376), *CACTA* (367), *Helitron* (77) *Tc1/Mariner* (37) and *hAT* (19). An association analysis permitted the identification of numerous TEs in the vicinity of genes (especially kinases, resistance genes and transcription factors) indicating their importance in the evolution of those regions. Our analysis provides a preview regarding the abundance and diversity of the transposable elements in cowpea.

Keywords: In silico analysis, mobile DNA sequences, legume evolution, *Vigna unguiculata*, *Glycine max*.

1 Introduction

The availability of plant genome sequences marked a decisive turn in our understanding of angiosperm genome content and evolution (Lopes *et al*., 2008). These sequences confirm that transposable elements (TEs) are among the most abundant components of the genome in higher plants, acting as recombination hot spots, allowing the acquisition of specific cell functions or still influencing protein-coding regions (Buisine *et al*., 2008; Tenaillon *et al.*, 2011). Therefore, the precise and comprehensive detection of TE sequences is a prerequisite for fully understanding their larger impact on genome structure, function, evolution as well as the role of TEs in shaping the regulatory network of eukaryotes (Collier and Largaespada, 2007; Flutre *et al*., 2011; Testori *et al.*, 2012). In addition, considerable attention has recently been given to transposable elements causing mutable alleles in plants for identification and isolation of gene. The transposon tagging has proven a powerful method to develop novel genetic tools for transformation and mutagenesis (Shimatani *et al*., 2009; Arensburger *et al*., 2011). Using transposons as tags, significant progress has been made in different plant species, including legumes (Hancock *et al*., 2011; Fladung and Polak, 2012).

There are two functional classes of transposable elements according to their transposition mechanisms: class I (retrotransposons), which move via a RNA intermediate in a 'copy-and-paste' fashion, and class II (transposons), which move via a DNA intermediate by a 'cut-and-paste' mechanism (Wicker *et al*., 2007). Class I members can be divided into several orders: long terminal repeat (LTR), dispersed nuclear element (DIRS), penelope-like element (PLE), short interspersed nuclear element (SINE) and long interspersed nuclear element (LINE). On the other hand, class II elements can be divided into two major subclasses: subclass 1 comprises classical 'cut-and-paste' TEs of the order TIR and subclass 2 holds DNA TEs that undergo a transposition process that entails replication without double-stranded cleavage, the rolling-circle *Helitrons* and the self-replicating *Maverick* (or *Polinton*)

elements (Feschotte *et al*., 2009). DNA transposable elements subclasses can be further split into superfamilies based on structural features and phylogenetic clustering. In plants, elements belonging to the *Tc1/Mariner*, *hAT*, *Mutator*, *PIF/Harbinger*, *CACTA* and *Helitrons* have been described (Feschotte and Pritham, 2007; Lal *et al*., 2009).

Computer-assisted analyses have provided information on the abundance (copy number), diversity (lineages), and temporal features of amplification for all major TE types (Holligan *et al*., 2006). The fundamental biological properties of TEs, including inter-species comparison, origin and interaction with host genomes may be inferred considering not only their structure but also their abundance and distribution in eukaryotic genomes (Quesneville *et al.*, 2005). Most TE analyses and classifications have been generally based on single species studies. Comparative studies have been rare, albeit more and more genome sequences from related species came to light in the past five years.

Legumes are major crop plants used in human food and animal feed due to the high seed protein content and their use as forage crops (Iantcheva *et al*., 2011). They can be divided into three subfamilies: Mimosoideae, Caesalpinioideae, and Papilionoideae (Doyle *et al*., 2003) with this last subfamily comprising most of the agriculturally important legumes, and also three important model species: *Medicago truncatula* L. and *Lotus japonicus* L. (both from the Galegoid clade), and *Glycine max* (L.) Merr. (soybean, from the Phaseoloid clade) (Cannon *et al.*, 2009). The ability to compare these genomes and to transfer information from these biological models to other crop species provides new insights for understanding legume genome organization and evolution.

The *Vigna* genus belongs to the Phaseoloid clade and the degree of conservation with *Glycine* is therefore expected to be relatively high. Indeed, Muchero *et al*. (2011) showed synteny between cowpea (*V. unguiculata*), *Glycine* and *Medicago* using mainly genic single nucleotide polymorphism (SNP) markers. The corresponding annotation and characterization of TE regions in cowpea has largely relied

upon comparison to *G. max* (Du *et al*., 2010) and *L. japonicus* (Holligan *et al*., 2006) Up to now, only class I TEs (*Ty1-copia* or *Ty3-gypsy*) have been characterized in cowpea (Galasso *et al*., 1997). Recently, over 250,000 genespace sequence reads (GSRs) from cowpea isolated by methylation filtering of genomic DNA have been released by Timko *et al*. (2008) with an average length of 610 base pairs (bp), representing about 160 Mb of genomic sequence information, opening the possibility for an in depth analysis of its TE content. Besides, there are 54,123 cowpea genome survey sequences (GSS) publicly available for download. In this report, we performed a systematic and fine-scale search of cowpea GRS and GSS to identify and characterize DNA transposons, using in silico procedures.

2 Materials and Methods

2.1 Transposon Mining and Sequence Availability

In this work we took the advantage of a recent published TE database for soybean (Du *et al*., 2010) to perform a comparative analysis using the cowpea Genespace. In the first step, we created a local data bank, in which 314,765 cowpea genome sequences were downloaded from NCBI and trimmed using a script developed in PERL and assembled by CAP3 using default parameters. Afterwards, we followed the evaluation protocol recommended by Wicker *et al*. (2007) with minor modifications to identify and classify class II TEs in cowpea. Further, we downloaded sequences of the class II TE from SoyTEdb.org (Soybean Transposable Element Database) for a tBLASTx search against the locally assembled cowpea genome sequences. Next, a BLASTn search was performed using the cowpea candidates retrieved from the initial tBLASTx search back to soybean class II TE, enabling a first homology-based classification of the tight related TE among cowpea and soybean. Following, a self BLASTn search was made with the now classified cowpea TEs, enabling the inclusion of more distantly

related TEs. The candidate sequences recovered from the tBLASTx search were also submitted to a BLASTx search against the nr-protein database at NCBI with automatic annotation by the program AutoFACT followed by visual curation. Figure 1 shows the pipeline applied for the TE annotation using cowpea genome sequences.

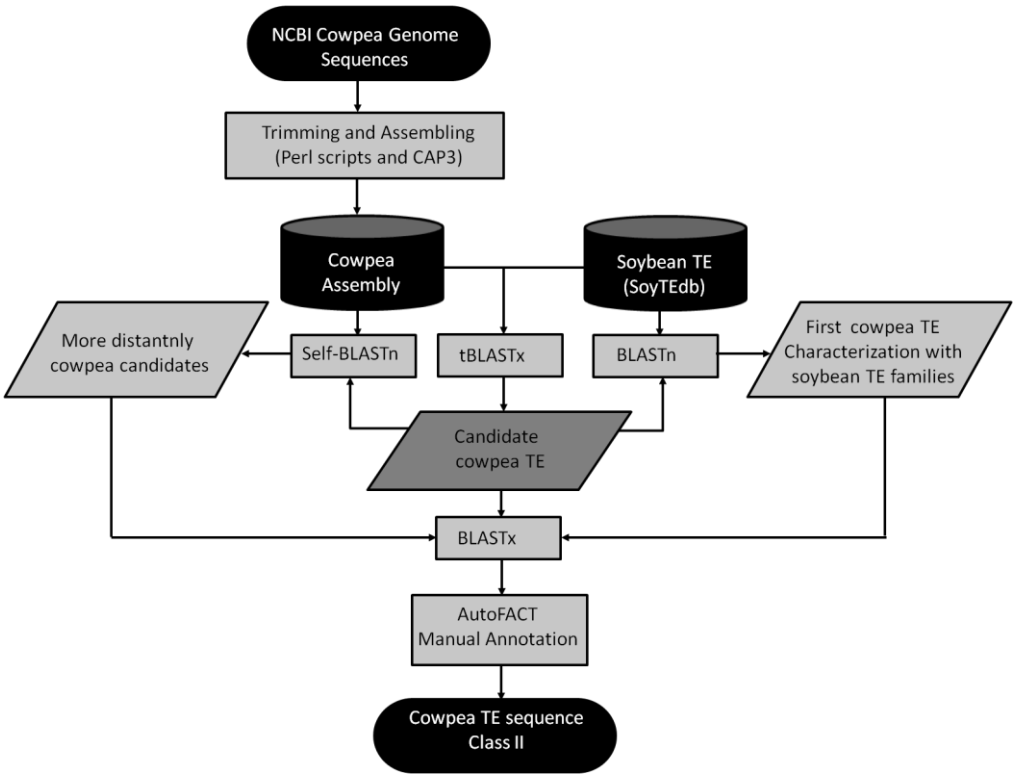


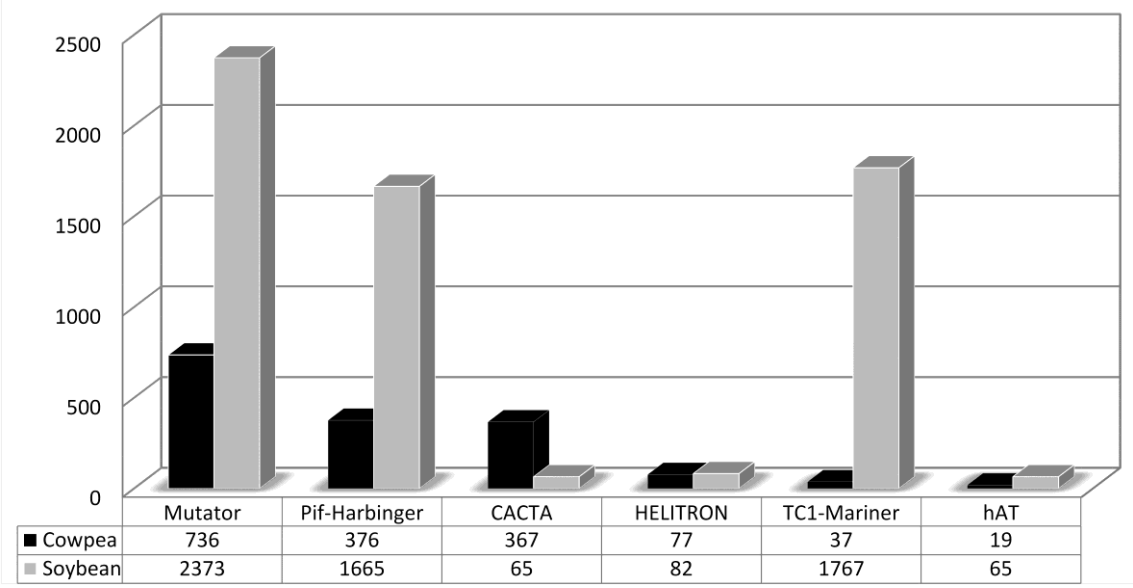
Figure 1 - A pipeline diagram showing the overall procedure for searching for the Class II transposable elements in cowpea genome sequences.

3 Results

Using computer-based sequence similarity searches, we systematically determined abundance of TE class II members in cowpea. After trimming, the public available sequences generated 310,060 valid sequences that could be assigned by CAP3 to 153,476 unique sequences including 50,101 contigs and 103,375 singlets. DNA transposons of six class II superfamilies were identified in the cowpea genespace sequences by homology with soybean DNA transposons, revealing a qualitative similarity among the genomes of cowpea and soybean, despite of the distinctive abundance of individual TEs (Figure 2).

In both species class II *Mutator* TEs were predominant, followed by *Tc1-Mariner* and *PIF-Harbinger* in soybean, whereas in cowpea *PIF-Harbinger* was the second most abundant, closely followed by *CACTA* transposons. In addition, the abundance of *Helitrons* DNA transposons was comparable in both species. Transposable elements of superfamilies *hAT* and *Tc1-Mariner* were the less abundant in cowpea.

The protein similarity screening against NCBI nr-protein databank revealed matches for 1,475 TE candidate sequences with at least one ORF of class II transposons. The BLASTx also enabled the identification of several gene classes in close vicinity (from 100 bp to 3 Kbp) to the identified TE candidates. The automatic annotation and the visual examination assigned that the most abundant genes linked to class II transposons were kinases, resistance (R) proteins and transcription factors (Figure 3).



	Mutator	Pif-Harbinger	CACTA	HELITRON	TC1-Mariner	hAT
Cowpea	736	376	367	77	37	19
Soybean	2373	1665	65	82	1767	65

Figure 2 - Comparison of the class II transposition elements (TE) content in *Vigna unguiculata* and *Glycine max*. The x-axis represents each TE type while the y-axis indicate TE amount for each class.

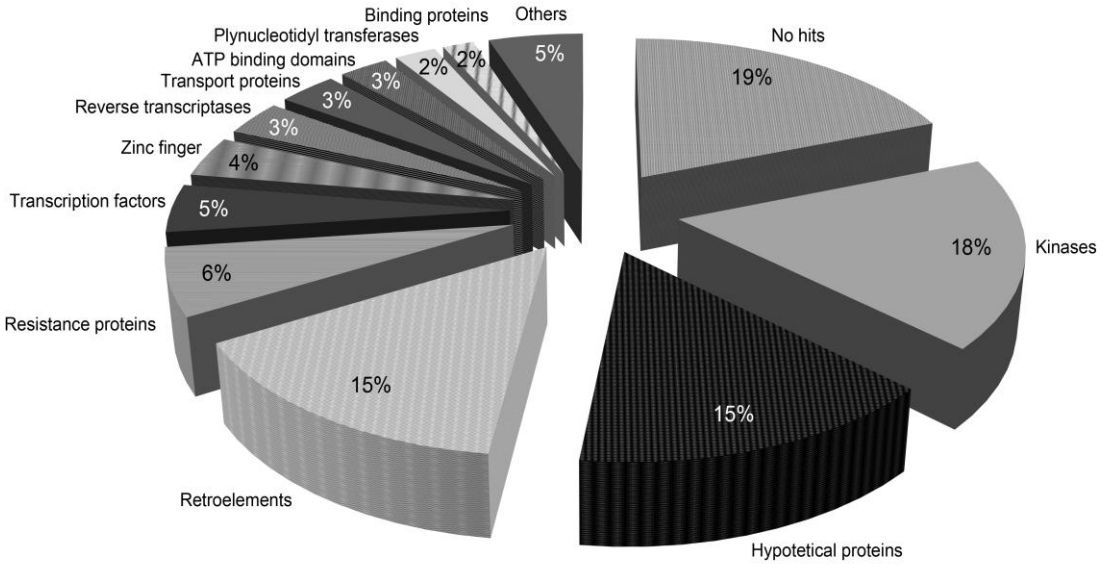


Figure 3 - Classification of 4,608 putative gene regions found in the vicinity of class II transposable element candidates in cowpea.

4 Discussion

Sequencing projects have been completed for a number of crop plants. However, for most species, only partial genome sequences are available. Despite the limited genome resources in cowpea, acess to most of the genes can be gained through genespace, which is the portion of the genome containing coding sequences, introns and cis-acting regulatory sequences (Muchero *et al.*, 2008). The genespace covers a narrow, 0.8–1.6%, GC (molar fraction of guanosine plus cytosine in DNA) range, probably because of the presence of abundant transposons elements (Barakat *et al*., 1997). These elements can produce changes in gene regulation and may even contribute protein domains, as in the cases of transposases that coopted as DNA binding proteins (Mach, 2010).

The transposon *Mutator* was first studied in maize, being considered one of the most active transposable elements described in plants. We have found 736 *Mutator*-like sequences in cowpea as compared with 2,373 elements previously described in soybean. Members of this superfamily are called *Mutator*-like elements (MULEs) which are characterized by long terminal inverted repeats (TIRs) that surround one or several open reading frames (Huan-Van and Capy, 2008). Chimerical MULEs carrying fragments of protein-coding genes are called Pack-Mules. In rice, it has been estimated that over 3,000 Pack-Mules contain genic fragments derived from more than 1,000 different cellular genes, whilst some of the captured genes might be functional (Jiang *et al.*, 2004). These elements were found to be abundant in *L. japonicus* where more than thousand members were identified (Holligan *et al*., 2006).

The high copy number of *Mutator* TEs in cowpea, soybean and *Lotus* indicates that this ‘cut-and-paste’ transposon is an early prevalent element within Papilionoideae, since *Lotus* belongs to the tribe Loteae that putatively diverged from the Phaseoleae (*Glycine*, *Phaseolus* and *Vigna*) at about 37-38 million years ago (Choi *et al*., 2004). The prevalence of *Mutator* elements over other groups of DNA-transposon was also observed in other plant genomes including bamboo, rice and maize, suggesting that

their differential proliferation substantially contributed to the genome size variation among these related species (Diao *et al*., 2006; Zhou *et al*., 2011). Most of the work on *Mutator* has been done in plants where they have been shown to have a variety of impacts on the evolution of the genomes they invaded (Marquez and Pritham, 2010) also revealing their heterogeneity that resulted in their classification into different subgroups according to their length and transposition properties (Leeuwen *et al*., 2006).

The *PIF-Harbinger* superfamily is the most recently discovered and widespread across fungal, plant and animal genomes (Zhang *et al*., 2004; Hancock *et al*., 2010) *PIF-Harbinger* elements have been shown to have undergone recent amplification in *Lotus* (Holligan *et al*., 2006) and *Medicago* (Grzebelus *et al*., 2007). Our results show that *PIF-Harbinger* elements have been significantly amplified in soybean and have attained a proportionally high copy number considering the available cowpea genomic sequences.

CACTA transposons are highly polymorphic and difficult to identify, but may be detected by the presence of a transposase-like protein. Interestingly, the *CACTA* elements identified in soybean were in much smaller number than in cowpea. However, the high diversity of the *CACTA* superfamily in soybean reported by Zabala and Vodkin (2008) may also have contributed to the low sequence similarity observed among both analyzed crops. *CACTA* are the most abundant class II elements in *Brassica oleracea* (Zhang and Wessler, 2004), and also in different *Oryza* (Zuccolo *et al*., 2007) and *Triticum* species (Charles *et al*., 2008), while they are much less abundant in *Arabidopsis* (Zhang and Wessler, 2004). These differences are not consistent with arguments suggesting that polyploids contain more TEs than diploid species (Soltis and Soltis, 1999). Benjak *et al*. (2008) suggested that grapevine databases contain a low number of EST sequences corresponding to the *CACTA* elements because most of them remain probably silent.Three transposon superfamilies are represented by a very small number of copies in the cowpea genome (*hAT*, *Tc1-mariner*, and *Helitron*). *Helitrons* are particularly abundant in

flowering plants, where they frequently acquire and sometimes express one or more gene parts (introns). Few *Helitrons* have been reported in *Medicago, Lotus* and rice. Apparently distinct eukaryotic genomes may accumulate a different number of *Helitrons*, being probably no major contributors to the genome size in any previously studied species (Yang and Bennetzen, 2009a). A more detailed analysis with intact *Helitron* sequences may show their contribution in genome size and if these elements acquired one or more gene fragments in cowpea and soybean genome.

Although *Tc1-Mariner* elements are present in cowpea, this species presents a lower copy number than soybean, similar to *L. japonicus* (Holligan *et al*., 2006). It was also consistent with the fact that the cowpea and *L. japonicas* genomes are smaller than that of soybean. Moreover, some comparisons have shown that the genome size is correlated with the amount of *Tc1-Mariner*. It is suggested that the *Tc1-Mariner* element presented no recent amplification in soybean, indicating that they may be inactive and relatively old in this species (Schmutz *et al.*, 2010). Opposite to that, in cowpea *Tc1-mariner* elements appear to have been active recently. In a recent review, Feschotte *et al.* (2007) reported that *Tc1-mariner* is widespread in plant genomes, in which they have given rise to a large fraction of plant MITEs.

The *hAT* transposon was represented by nineteen sequences in cowpea and sixty-five sequences in the soybean genome. Since *hAT* elements are relatively ancient in eukaryote genomes (Rubin *et al*., 2001), a high proportion of defective *hAT* elements is expected. Thus, *hAT* elements may have been frequent invaders of both cowpea and soybean genomes in the past, producing abundant nonautonomous families but failing to become stable residents of theses genome.

4.1 Cowpea transposable elements were detected in the vicinity of genes

The protein similarity screening against nr-proteins from NCBI showed matches for 1,475 TE candidate sequences presenting at least one ORF of class II transposons. The BLASTx also enabled the

identification of several gene classes (Figure 3) in close vicinity to the TE candidates. The automatic annotation confirmed by visual examination assigned that the most abundant genes linked to transposons of class II were kinases, resistance genes and transcription factors. Regarding the kinases, 826 sequences could be assigned, indicating the influence of TEs in the evolution of this gene class responsible by the transmission and amplification of defense signal cascades (Xu *et al*., 2010). Similarly, a survey of a BAC library from melon sequenced by Gonzáles *et al.* (2010) revealed that almost half of the melon specific transposons were interspersed with NBS-LRR (nucleotide-binding site–leucine-rich repeats that are associated to kinase domains) genes, that potentially form resistance gene clusters.

It is known that TEs contribute significantly to genomic plasticity in the face of diverse environmental conditions (Wang *et al*., 2003). In plants, it has long been hypothesized that transposable elements play an important role in *R*-gene evolution (Lee *et al*., 2009). In rice, for example, transposon-like elements appear to be a major source of variability of the *Xa21*-gene family members. For example, fourteen transposon-like elements grouped into 11 families, including three families of MITEs, *Ds*-like elements, a *CACTA*-like element and a retrotransposable element at the rice *Xa21* locus (Song *et al*., 1998). Transposons were also found in *N* resistance genes in flax, *RPP5* homologues in *Arabidopsis thaliana*, and *R1* homologues in potato (Noël *et al*., 1999; Dodds *et al*., 2001; Kuang *et al*., 2005).

The association of TEs with kinases and resistance genes, observed in the present work is interesting, since regions rich in those gene families are known as "fast evolving", with evolutionary hotspots especially due to the known plant-pathogen co-evolution pressure (Dodds and Rathjen, 2010), a fact recognized in many *R* gene rich regions previously evaluated. The putative role of the transposable elements in the divergent evolution of such regions is attributed to insertion events that lead to unequal crossing over events and consequent duplication/deletion events (reviewed by Michelmore and Meyers, 1998).

Also the presence of such TEs in the of vicinity transcription factors was observed in both *Arabidopsis* and rice, where cellular genes encoding DNA-binding and transcription factors appear to have frequently been the targets of transduplication, especially concerning MULE transposons (Juretic *et al*., 2005). The high number of genes in close vicinity to the TE candidates observed in this study may confirm previous presumptions that they are not randomly distributed in eukaryote genomes, with some TE classes presenting putative preferential integration in gene-rich regions (Gonzáles *et al.*, 2010). It is known that DNA transposons from several different superfamilies may capture gene fragments. In maize, for example, *Helitrons* acquire and carry fragments of genes from different locations in the host genome through a duplicative mechanism, referred to as transduplication (Lai *et al*., 2005; Yang and Bennetzen, 2009b). Pack-MULEs bear the ability to capture genes or gene fragments from several loci and move them around the genome, a feature that confers these TEs a potential role in gene evolution in rice, melon (Gonzáles *et al.*, 2010), *Arabidopsis* (Jiang *et al*., 2011) and *Lotus* (Holligan *et al*., 2006). The protein hits of Pack-MULEs in plants include a variety of functional domains such as kinases, transcription factors and transporters. Similarly, *CACTA* elements were reported capturing gene fragments from soybean (Zabala *et al.*, 2005) and *Antirrhinum* (Roccaro *et al.*, 2007).

5 Final considerations

The present work revealed a considerable diversity of class II transposable elements in cowpea, as compared with those known from soybean. The association of the transposable sequences found in the cowpea genespace to regions bearing genes responsible for signal transduction and pathogen resistance indicate a putative role in the evolution of those regions. Furthermore, this association may be useful for the development of molecular markers for genetic mapping in cowpea, allowing a sensitive

polymorphism detection with potential for future marker assisted selection useful for cowpea breeding projects and maybe also for applications regarding other related *Vigna* species.

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CAPÍTULO IV
Artigo em Fase de Conclusão –
A ser Submetido à Revista Chromosome Research

Ocorrência e distribuição dos retrotransposons Ty1-*copia*-like e Ty3-*gypsy*-like em espécies dos gêneros *Glycine* Willd., *Phaseolus* L. e *Vigna* Savi.

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Resumo

O domínio conservado dos genes RT (*Reverse Transcriptase*; Transcriptase Reversa) dos retrotransposons LTR Ty1-*copia*-like e Ty3-*gypsy*-like foram amplificados a partir do genoma de *Vigna unguiculata* usando *primers* degenerados e hibridizados *in situ* em alguns representantes dos gêneros *Glycine* L., *Phaseolus* L. e *Vigna* Savi. A FISH (*Fluorescent In Situ Hybridization*; Hibridização *in situ* Fluorescente) evidenciou a presença de sinais dispersos e pericentroméricos, utilizando ambos retroelementos, em *Glycine max, Phaseolus vulgaris, P. lunatus, V. unguiculata* e *Vigna radiata*, com algumas divergências interespecíficas. Tais marcações estavam aparentemente associadas com loci de DNAr 5S e 45S, bem como distribuídas em regiões heterocromáticas pericentroméricas e subterminais, enfatizando uma distribuição preferencial nos genomas das leguminosas analisadas. A grande abundância das sequências RT foi atribuída à alta taxa de mutação e à baixa pressão seletiva, associadas ao mecanismo de retrotransposição do tipo *copy and paste* específico destes retroelementos, resultando em um acúmulo dos elementos transponíveis em regiões preferenciais, como heterocromatina e loci de DNAr, ao longo da diferenciação cariotípica das espécies analisadas.

Palavras-chave: Ty1-*copia*-like, Ty3-*gypsy*-like, FISH, leguminosas

Abstract

The conserved domains of reverse transcriptase (RT) genes of *Ty1-copia* and *Ty3-gypsy* groups of long terminal repeat (LTR) retrotransposons were amplified from *Vigna unguiculata* genome using degenerate primers and located *in situ* in representatives of *Glycine* L., *Phaseolus* L and *Vigna* Savi genus. FISH analysis has showed the presence of dispersed and pericentromeric signals, using both retroelements, in *Glycine max, Phaseolus vulgaris, P. lunatus, V. unguiculata* and *Vigna radiata*, with some interspecific divergences. Such marks were apparently associated, in some cases, to 5S and 45S rDNA loci, and located in pericentrometric and subterminal heterochromatic regions, emphasizing a preferential distribution in the analyzed genomes. The large occurrence of RT sequences within genomes and their differential distribution along chromosomes should be due to the high mutational rates and low selective pressure associated to the *copy and paste* retrotransposition mechanism, affecting the structural evolution of plant genomes, and especially of these legumes.

Keywords: Ty1-*copia*-like, Ty3-*gypsy*-like, FISH, Leguminosae

Introdução

Os retrotransposons representam uma importante fração do DNA moderadamente repetitivo, apresentando um mecanismo de transposição do tipo *copy and paste* via RNA intermediário ao longo dos genomas, o qual é apontado como o principal fator responsável pela expansão e evolução dos genomas vegetais (Todorovska, 2007; Du *et al*., 2010a).

Estes retroelementos são subdivididos em duas subclasses, as quais diferem em sua estrutura e ciclo de transposição, incluindo retroelementos LTRs (*Long Terminal Repeats*) and não-LTRs (Friesen *et al*., 2001; Park *et al*., 2007). Entre os retrotransposons LTRs, os elementos Ty1-*copia* (Pseudoviridae) e Ty3-*gypsy* (Metaviridae) são considerados os mais abundantes e relatados nos genomas de diferentes angiospermas, representando, 25% e 75% dos genomas de arroz e milho, respectivamente (Du *et al*., 2010a).

Os dois elementos diferenciam-se pelo domínio conservado dos genes RT, característico de cada retroelemento (Friesen *et al*., 2001), bem como pela ordem gênica dos domínios RT e integrase na região *pol*. O Ty1-*copia* apresenta o domínio da integrase anterior (posição 5') ao da RT e RNaseH, enquanto que no Ty3-*gypsy*, tal domínio situa-se posterior (posição 3') ao RT e RNAseH. Adicionalmente, o Ty3-*gypsy* apresenta uma maior similaridade com os retrovírus (Kumar e Bennetzen, 1999; Hansen e Heslop-Harrison, 2004), pela presença de um domínio *env* (Envelope), coincidente com a posição ocupada pelo gene codificante do envelope viral em retrovírus (Vershinin *et al*., 2002), sendo estes classificados como *Gypsy-like* (Capy *et al*., 1998).

Diferentes estudos têm isolado e caracterizado o domínio RT de famílias de retrotransposons, observando a distribuição destes elementos no genoma. Os retroelementos Ty1-*copia* e Ty3-*gypsy* têm prevalecido nos principais grupos vegetais, como em *Helianthus* L. (Natali *et al*., 2006; Ungerer *et al*., 2009), *Malus domestica* Borkh (Sun *et al*., 2008), *Pisum sativum* L. (Macas *et al*., 2007). Entretanto, Dixit *et al*. (2006) relataram que a ocorrência de Ty3-*gypsy* tem sido pouco identificada, estando limitada a alguns grupos taxonômicos (Natali *et al*., 2006; Park *et al*., 2007).

A elevada abundância e taxa de movimentação genômica têm permitido a utilização dos retroelementos na FISH (*Fluorescent In Situ Hybridization*), em espécies proximamente relacionadas, contribuindo para o melhor entendimento de relações filogenéticas (Nielen *et al*., 2009). Os gêneros *Glycine* Willd., *Phaseolus* L. e *Vigna* Savi pertencem à tribo Phaseoleae, agrupados em um clado monofilético (Phaseoloids), assim como apresentam ampla distribuição mundial (Marechal *et al*., 1978). Representantes destes gêneros apresentam grande importância econômica, destacando-se as espécies *G.*

max (L.) Merr., *P. vulgaris* (L.), *P. lunatus* (L.) e *V. unguiculata* (L.) Walp conhecidas popularmente como soybean, common bean, Lima bean and cowpea, respectively.

Considerando a existência de poucos trabalhos que caracterizem a organização genômica de elementos retrotransponíveis em leguminosas cultivadas, o presente trabalho caracterizou sequências RT dos elementos Ty1-*copia*-like e Ty3-*gypsy*-like do genoma de *V. unguiculata*, bem como, realizou uma abordagem comparativa da localização cromossômica dos citados retroelementos em espécies dos gêneros *Glycine*, *Phaseolus* e *Vigna* visando uma melhor compreensão sobre a distribuição do DNA repetitivo e o entendimento da evolução cariotípica na tribo Phaseoleae.

Material e métodos

Material vegetal

Sementes das espécies *Glycine max* cv. Conquista, *P. vulgaris* cv. BRS Esplendor, *P. lunatus* cv. Vermelhinha, *V. unguiculata* cv. BR14-Mulato e*Vigna radiata* cv. Berken foram obtidas do banco de germoplasma da Embrapa Soja (Empresa Brasileira de Pesquisa Agropecuária, Londrina), Embrapa Arroz e Feijão (Santo Antônio de Goiás), para feijão comum e feijão-fava , Embrapa Meio-Norte (Teresina) e do Jardim Botânico de Kew (Royal Botanic Gardens), para feijão-caupi ee feijão-mungo, respectivamente.

Análise molecular

Isolamento e caracterização do domínio da transcriptase reversa (RT) dos retrotransposons Ty1-*copia*-like e Ty3-*gypsy*-like

A extração do DNA genômico foi realizada a partir de folhas jovens de *V. unguiculata* BR14-Mulato utilizando o método de CTAB, segundo descrito por Weising *et al*. (1995). Posteriormente, a obtenção da região genômica correspondente à transcriptase reversa do retrotransposon Ty1-*copia*-like foi realizada pela amplificação de DNA por PCR (*Polymerase Chain Reaction*; Reação da Polimerase em Cadeia) utilizando os *primers* degenerados 5'-GAGAATTCACNGCNTTYYTNCAYGG-3' e 5'-GAGGATCCATRTCRTCNACRTANAR-3' (Xiao *et al*., 2004), desenhados para os motivos conservados 5'-TAFLHG-3' e 5'-YVDDML-3' da referida enzima. Por sua vez, o elemento Ty3-*gypsy*-like foi obtido pela amplificação com os *primers* GyRT1 (RMCVDYR, 5'-

MRNATGTGYGTNGAYTAYMG-3') e GyRT4 (YAKLSKC, 5'-RCAYTTNSWNARYTTNGCR-3') (Friesen *et al*., 2001).

Os produtos de PCR foram examinados em gel de agarose 1,8% corado com brometo de etídeo (0,5 mg/mL), e analisados sob luz ultravioleta em transiluminador. Os fragmentos de interesse amplificados foram excisados do gel de agarose com auxílio de um bisturi descartável, sendo purificados mediante o kit comercial GFX PCR DNA e Gel Band Purification Kit (GE Healthcare, USA), de acordo com especificações fornecidas pelo fabricante.

Clonagem e sequenciamento dos fragmentos RT

Os fragmentos purificados foram clonados no vetor pGEM® -T Easy (Promega), o qual foi inserido em *Escherichia coli* (cepa DH10B) por eletroporação, utilizando o BioRad - modelo Gene Pulser II.

A confirmação do inserto de interesse nos plasmídeos recombinantes foi verificada por PCR de colônia, utilizando os *primers* T7 e SP6 que anelam em regiões do vetor pGEM-T Easy. Clones positivos foram selecionados, sendo os DNA plasmidiais extraídos utilizando o protocolo do Qiagen Plasmid Mini Kit (Qiagen), com algumas adaptações. Para as reações de sequenciamento dos clones recombinantes foi utilizado o *kit BigDye Terminator* versão 3.1 (*Applied Biosystem*), os *primers* M13 (Fw): 5'- GTAAAACGACGGCCAGTG -3' e M13 (Rv): 5'- GGAAACAGCTATGACCATG-3' e o equipamento automático *ABI 3100* (*Applied Biosystem*).

As sequências foram analisadas mediante o *software* BioEdit 7 (Hall, 1999), visando à construção de sequências consenso, as quais foram comparadas com os dados depositados no *GenBank* utilizando o programa BLAST (*Basic Local Alignment Search Tool*) (Altschul *et al*., 1997) (www.ncbi.nlm.nih.gov/BLAST), sendo a seguir analisadas com o programa ORF Finder para identificação de domínios conservados. As análises subsequentes de nucleotídeos e proteínas foram realizadas utilizando o programa CLUSTAL W (Thompson *et al*., 1994), sendo necessários alguns ajustes manuais nos alinhamentos.

Estudos citogenéticos

A análise mitótica foi realizada utilizando pontas de raízes pré-tratadas com uma solução de 8-hidroxiquinoleína (8HQ) por 18 h, 1 h à temperatura ambiente e 17 h a 8 ºC, fixadas em etanol absoluto:ácido acético (3:1, v/v) ou metanol:ácido acético (3:1, v/v) por 2 a 24 h à temperatura ambiente e estocadas a -20 ºC. Para a preparação das lâminas, os meristemas foram lavados duas vezes em água destilada, por 5 min, digeridos em solução enzimática contendo 2% (p/v) celulase 'Onozuka R-10' (Serva) e 20% (v/v) pectinase (Sigma-Aldrich) em tampão citrato 10 mM por 180 min a 37 °C. Em seguida, o material digerido foi lavado em água destilada e incubado em ácido acético 45% por 30 min a 37 °C. Uma ponta de raiz foi utilizada por lâmina, a qual foi macerada em ácido acético 45%, sendo posteriormente submetida a nitrogênio líquido para remoção da lamínula com posterior secagem ao ar. Outra metodologia utilizada seguiu o procedimento descrito por Carvalho e Saraiva (1993), com algumas modificações.

Em ambos os casos, as lâminas foram selecionadas mediante coloração com uma solução de 2 μg/mL de DAPI (4´-6´-diamidino-2-fenilindole)/ glicerol (1:1, v/v). As preparações selecionadas foram descoradas em etanol absoluto:ácido acético (3:1, v/v) e etanol absoluto por 30 min e 1 h, respectivamente. Após secagem ao ar, as lâminas foram devidamente estocadas a -20 °C, até o momento do procedimento da FISH.

Marcação e obtenção das sondas de retrotransposons e DNAr 5S e 45S

Os fragmentos Ty1-*copia*-like e Ty3-*gypsy*-like isolados do genoma de *V. unguiculata* foram marcados com digoxigenina-11-dUTP (Roche) por *nick translation* (Invitrogen). Sondas R2, um fragmento de 6,5 Kb, contendo a unidade de repetição de DNAr 18S-5,8S-25S, oriunda de *Arabidopsis thaliana* (L). Heynh (Wanzenböck *et al*., 1997), e D2, um fragmento de 400 pb contendo duas unidades de repetição de DNAr 5S, proveniente de *Lotus japonicus* (Regel) K.Larsen (Pedrosa *et al*., 2002), também foram utilizadas como marcadores cromossômicos. As sondas de DNAr 5S e 45S foram marcadas com biotina-11-dUTP (Sigma) e digoxigenina-11-dUTP, respectivamente, por *nick translation* .

Hibridização in situ Fluorescente (FISH)

As lâminas, estocadas a -20 ºC, foram pré-tratadas como descrito por Pedrosa *et al.* (2001). A desnaturação das sondas, os banhos pós-hibridização e a detecção foram efetuados de acordo com Heslop-Harrison *et al.* (1991), exceto pela lavagem de estringência a qual foi realizada em 0,1xSSC a 42 ^{o}C. As misturas de hibridização consistiram de formamida 50% (v/v), dextran sulfato 10% (p/v), 2x SSC e 2-5 ng/μL de sonda. As lâminas foram desnaturadas por 7 min a 78 ^{o}C, bem como hibridizadas por 18 a 48 h a 37 °C. Para soja, os cromossomos foram desnaturados com formamida 70% em 2xSSC a 70 ºC por 7 min, sendo posteriormente, desidratados por 5 min em uma série alcoólica (70% e 100%) a -20 ºC. As sondas marcadas com biotina foram detectadas com antibiotina obtida em camundongo (Dako) em combinação com anticamundongo conjugado com TRITC (Dako) em BSA 1% (p/v). Enquanto as sondas marcadas com digoxigenina foram detectadas usando antidigoxigenina de ovelha conjugado com fluoresceína isotiocianato (FITC; Roche) e amplificadas com antiovelha conjugado com FITC (Sigma) também em BSA 1% (p/v). Todas as preparações foram montadas com 2 μg/mL de DAPI em Vectashield (Vector).

Análise dos dados

As células foram analisadas com um microscópio de epifluorescência Leica DMLB e imagens das melhores células foram capturadas com uma câmera Leica DFC 340FX, utilizando o programa CW 4000 da Leica. As imagens foram otimizadas para melhor brilho e contraste com o Adobe Photoshop CS4 (Adobe Systems Incorporated). Para as imagens sobrepostas DAPI-DNAr 45S, a imagem do DAPI foi transformada para tons de cinza, o DNAr foi pseudocolorido com amarelo e ambas as imagens foram sobrepostas, usando a ferramenta *lighten*.

Para a identificação cromossômica de *P. vulgaris* e *P. lunatus* seguiu-se a nomenclatura determinada por Pedrosa-Harand *et al*. (2008) e Almeida (2006), respectivamente. Para *V. unguiculata* e *V. radiata*, foram utilizadas as identificações descritas por Bortoleti *et al.* (2012).

Resultados

Isolamento do domínio RT de sequências Ty1-*copia*-like e Ty3-*gypsy*-like do genoma de *V. unguiculata*

A PCR com os *primers* degenerados para o domínio RT do retrotransposon Ty1-*copia*-like produziu apenas um fragmento com o comprimento de aproximadamente 270 pb (Figura 1A), o qual foi purificado e clonado. O clone apresentou um inserto com o tamanho de 279 pb (Figura 2A), o qual apresentou similaridade com os fragmentos RT dos retroelementos Ty1-*copia*-like das espécies *V. radiata*, *Camellia sinensis*, *Orobanche crenata* e *Solanum tuberosum*, ressaltando-se a presença dos motivos LYGLKQ e LLYVDDM nas respectivas sequências (Figura 2B). Foi acrescentada a sequência de *V. unguiculata* do trabalho de Galasso *et al.* (1997).

Por sua vez, a reação inespecífica com os *primers* degenerados GyRT1 e GyRT4 produziu bandas com tamanhos variáveis (Figura 1B), sendo isolado e purificado o fragmento correspondente a aproximadamente 430 pb. Após sequenciamento, o clone apresentou um inserto com o tamanho de 417 pb (Figura 2C), cuja sequência mostrou similaridade com fragmentos do domínio RT dos retroelementos Ty3-*gypsy*-like caracterizados em *V. radiata*, *Prunus mune*, *O. crenata*, *Phelipanche tunetana* e *Elaeis guineesis*, sendo notado a conservação dos motivos MCVDYR, MPFGV e YAKL-KC em tais sequências (Figura 2D).

Distribuições das sequências RT de retroelementos Ty1-*copia*-like e Ty3-*gypsy*-like mediante FISH

A FISH com os domínios RT dos retroelementos Ty1-*copia*-like (*Vu* 03 clone) e Ty3-*gypsy*-like (*Vu* 02 clone) resultou em marcações dispersas ao longo dos complementos cromossômicos de *G. max*, *P. vulgaris*, *P. lunatus*, *V. unguiculata* e *V. radiata* (Figuras 3 e 4).

As espécies *G. max*, *P. vulgaris* e *P. lunatus* apresentaram padrões similares de distribuição do retroelemento Ty1-*copia*-like, com marcações dispersas ao longo dos pares cromossômicos. Contudo, em alguns cromossomos, foram visualizados sinais mais evidentes nas regiões pericentroméricas (Figura 3A', B'e C'). Uma aparente associação foi observada entre as marcações do retrotransposon com os sítios de DNAr 5S no par cromossômico 19 em soja (Figura 3A'), assim como no 6 e 10 em feijão

comum (Figura 3B’). Por sua vez, os sinais de DNAr 45S encontraram-se adjacentes às marcações do Ty1-*copia*-like na maioria dos taxa supracitados (Figura 3A, B e C).

Em *V. unguiculata*, as marcações foram mais concentradasou nas regiões intercalares e subteloméricas dos cromossomos (Figura 3D’), estando aparentemente associados aos sítios de DNAr 45S observados nos pares cromossômicos B, D, F, G e J (Figura 3D). Tal associação também foi notada para os sítios de DNAr 5S localizados no par cromossômico D (Figura 3D’). No feijão-mungo, uma marcação dispersa foi visualizada em todo o complemento cromossômico para o retroelemento (Figura 3E’), notando-se uma aparente associação com os sinais de DNAr 45S localizados no par cromossômico K da referida espécie (Figura 3E).

Similarmente ao notado para a sequência Ty1-*copia*-like, marcações pericentroméricas do retrotransposon Ty3-*gypsy*-like foram encontradas, juntamente com sinais dispersos, ao longo dos cromossomos de *G. max* (Figura 4A'). Em *P. vulgaris*, uma disposição dispersa para maioria dos cromossomos foi evidente para os sítios de Ty3-*gypsy*-like; entretanto, alguns sinais foram visualizados em posição intercalar e terminal (Figura 4B’), estando os sítios de DNAr 45S adjacentes às marcações deste retroelemento nos pares cromossômicos 6, 9 e 10 da referida espécie (Figura 4B’).

No feijão-fava, marcações dipersas foram observadas para o retroelemento Ty3-*gypsy*-like, contudo, alguns cromossomos apresentaram sinais pericentroméricos bem evidentes. Adicionalmente, o par cromossômico 10 apresentou uma marcação bem característica envolvendo a extensão de seu braço longo (Figura 4C’). Com relação às espécies *V. unguiculata* e *V. radiata*, destacam-se marcações dispersas ao longo da maioria dos cromossomos (Figura 4D’ e E’), aparentemente colocalizadas com os sítios de DNAr 45S (Figura 4D e E).

Discussão

O isolamento e amplificação dos domínios RT dos retroelementos Ty1-*copia*-like e Ty3-*gypsy*-like do genoma de *V. unguiculata*, caracterizados pela presença de motivos específicos (Flavell *et al*., 1992; Sun *et al*., 2008), confirmaram a especificidade dos *primers* degenerados para esta finalidade, ressaltando o elevado nível de conservação do domínio RT em diferentes organismos vegetais, como relatado por Doolittle *et al*. (1989).

Este elevado nível de conservação pode ser suportado pelo processo de transferência vertical, uma vez que postula a existência de um ancestral comum com retroelementos similares, os quais foram transmitidos durante os eventos de especiação subsequentes, da linhagem parental para a progênie,

juntamente com outros componentes do genoma. Tal mecanismo de transferência tem sido relatado para elementos Ty1-*copia*-like em briófitas, pteridófitas, angiospermas e gimnospermas (Flavell *et al*., 1992; Voytas *et al*., 1992; Kumar *et al*., 1997).

Por outro lado, alguns estudos sugerem que a transmissão horizontal tem desempenhado um papel importante na evolução e dispersão dos retrotransposons entre diferentes espécies vegetais (Flavell *et al*. 1992; Cheng *et al*., 2009). Todavia, a identificação de sequências semelhantes dos retroelementos Ty1-*copia*-like e Ty3-*gypsy*-like em diferentes espécies seria necessária para explicar como esses elementos movem-se entre espécies isoladas reprodutivamente.

Adicionalmente, a conservação do domínio RT permitiu a utilização deste fragmento dos retrotransposons, Ty1-*copia*-like e Ty3-*gypsy*-like, como sonda na FISH auxiliando na localização cromossômica desses elementos nos genomas das espécies analisadas. A análise da distribuição cromossômica mostra-se de grande valia para o entendimento do processo de expansão dos retrotransposons em diferentes espécies proximamente relacionadas, uma vez que esses elementos constituem uma fração bem representativa dos genomas vegetais (Lamb e Birchler, 2006).

Geralmente os retrotransposons apresentam uma organização cromossômica dispersa, a qual reflete o modo de amplificação e inserção desses elementos, sendo encontrados entre outras sequências repetitivas ou associados a regiões genômicas particulares (Kubis *et al*., 1998; Heslop-Harrison, 2000). Em todas as espécies analisadas, um padrão de distribuição disperso foi notado para o retroelemento Ty1-*copia*-like, fato comum descrito anteriormente para várias espécies, como *Beta vulgaris* L. (Weber *et al*., 2010), *Copaifera langsdorffii* Desf. (Gaeta *et al*., 2010) e *Helianthus annuus* L. (Natali *et al*., 2006), enfatizando a afirmação que este grupo corresponde aos mais bem caracterizados retrotransposons em vegetais (Todorovska, 2007). Por outro lado, esses sinais dispersos mostraram uma aparente associação com os loci de DNAr 5S (em *G. max* e *P. vulgaris*) e 45S (em *V. unguiculata* e *V. radiata*), bem como com as sequências de DNA satélite constituintes da heterocromatina terminal e pericentromérica, presentes nos genomas das leguminosas estudadas, sugerindo uma localização preferencial entre tais sequências.

Entretanto, tais dados contradizem a afirmação de que os elementos Ty1-*copia*, geralmente estão presentes em ambos os braços cromossômicos, com a exclusão dos centrômeros, telômeros e loci de DNAr 45S (Weber *et al.*, 2010), como observado anteriormente, em *V. unguiculata* por Galasso *et al*. (1997). Provavelmente, esses autores amplificaram e hibridizaram o domínio RT pertencente a uma família de Ty1-*copia* diferente da amplificada no presente trabalho, o que justificaria as divergências em

termos de sequência de DNA e/ou organização genômica, salientando que é possível a presença de diferentes famílias no genoma de um organismo, tratando-se de uma característica geral das angiospermas (Fregonezi *et al*., 2007).

Em *G. max*, a localização pericentromérica de sítios do Ty1-*copia*-like e Ty3-*gypsy*-like foi evidente, assim como coerente com os dados resultantes do sequenciamento genômico da referida espécie. Aproximadamente 42% do genoma da soja é constituído por retrotransposons do tipo LTR, compreendendo mais de 32.000 elementos, os quais estão depositados no banco de dados SoyTEdb (Du *et al.,* 2010b). Entre esses, foi estimada a presença de 510 famílias diferentes de retrotransposons distribuídas pericentromericamente, contendo 14106 elementos completos cujo tamanho variou de 1 a 21 Kb, sendo 31% e 69% classificados como *copia-like* e *gypsy-like*, respectivamente (Du *et al*., 2010a; Schmutz *et al*., 2010).

No presente trabalho, os sinais Ty3-*gypsy*-like visualizados, também, na região proximal dos cromossomos das espécies analisadas, indicam que as sequências desse retroelemento não estão agrupadas, mas dispersas, conforme observado para o Ty1-*copia*-like. Supostamente, estes sítios dos retroelementos LTR fazem parte da heterocromatina pericentromérica ressaltada pelo padrão de bandeamento CMA_3/DAPI em *G. max* (CMA_3^+), *V. unguiculata* (CMA_3^+), *V. radiata* ($DAPI^+$) (Bortoleti *et al*., em preparação), bem como em *P. vulgaris* e *P. lunatus* (CMA_3^+) descrito por Almeida (2006).

A distribuição dos retroelementos Ty3-*gypsy*-like tem sido menos estudada, em comparação ao Ty1-c*opia*-like (Natali *et al*., 2006, Fregonezi *et al.,* 2007). Por acumularem-se preferencialmente nas regiões centroméricas (Weber *et al*., 2010), a maioria destas sequências vem sendo denominada CRs (retrotransposons centroméricos) em diferentes grupos vegetais, como por exemplo, *Brassica* L. (Lim *et al*., 2007), *Glycine* L. (Tek *et al.,* 2010), *Lotus* L. e *Medicago* L. (Gorinsĕk *et al*., 2004), apresentando um elevado nível de conservação o que sugere a ocorrência de mecanismos de transposição recentes deste retroelemento, bem como seu papel essencial na manutenção e funcionamento do centrômero (Nagaki *et al*., 2003; Neumann *et al*., 2011).

A presença de retrotransposons em regiões heterocromáticas tem sido relatada (Dimitri *et al.*, 1999), sugerindo que esta classe de cromatina pode tolerar o acúmulo de elementos funcionais ou degenerados, uma vez que, geralmente, caracteriza-se como uma região genômica silenciada, acompanhada por metilação de DNA e modificações de histonas (Natali *et al.,* 2006; Weber *et al*., 2010). Adicionalmente, o envolvimento da pressão seletiva contra a inserção dos retroelementos em regiões codificantes favorece esta localização preferencial na heterocromatina. A inserção em genes não

é desejada, pois pode causar efeitos deletérios para a estrutura e o funcionamento de genes, modificações no padrão espacial e temporal da regulação gênica, bem como a origem de diferentes rearranjos cromossômicos (Rajput e Upadhyaya, 2009).

Contudo, vale salientar que a associação preferencial heterocromática não é obrigatória; por exemplo, em *Arachis hypogaea* L., foi relatada a presença do retroelemento Ty3-*gypsy*-like (tipo *Athila*) em regiões eucromáticas, estando tais elementos ausentes nas regiões teloméricas e centroméricas (Nielen *et al*., 2009). Em *P. vulgaris*, a família Tpv2 (Ty1-*copia*) apresentou os seus sítios de integração localizados em (ou próximos a) regiões transcricionalmente ativas (Garber *et al*., 1999), enfatizando uma distribuição preferencial de integração na eucromatina.

A aparente associação de sítios de retroelementos Ty1-*copia* e de DNAr 5S e 45S mostrou-se uma característica peculiar em algumas leguminosas estudadas, fato também observado em *Hordeum spontaneum* (K.Koch) Thell (Belyayev *et al*., 2005), *Cestrum strigilatum* Ruiz & Pav. e *C. intermedium* Sendtn. (Fregonezi *et al*., 2007) para o DNAr 45S. Em *P. vulgaris*, os dois pares cromossômicos portadores de DNAr 5S apresentaram associação com sítios de Ty1-*copia*-like. Entretanto, essa homogeneidade não foi observada em *V. unguiculata* e *V. radiata* para os loci de DNAr 5S e 45S, respectivamente. No feijão-caupi, o par cromossômico I não apresentou associação entre os sinais do retroelemento e DNAr 5S, conforme foi observado para o cromossomo D; enquanto que, em feijão-mungo, não foi visualizada a associação de marcações Ty1-*copia*-like e DNAr 45S no par cromossômico F, conforme observado para o cromossomo K, também portador deste locus. Possivelmente, tais divergências estejam relacionadas com diferenças nas taxas de homogeinização e amplificação dos retroelementos, bem como com o índice de frequência de recombinação ocorrente nestas regiões cromossômicas.

Os loci de DNAr têm sido apontados também como sítios preferenciais para a inserção de elementos móveis, os quais são eliminados pelo processo de *crossing-over* desigual entre cromátides-irmãs, conforme o modelo de evolução em concerto característico dos genes de RNAr, evitando alteração ou inativação dos genes de RNAr. Assim sendo, a evolução em concerto permite que os loci de DNAr funcionem homogeneamente em face ao ataque contínuo de inserções destes elementos, além de favorecer a ativação transposicional, visto que facilita a interação dos retroelementos com vários sítios alvos (Eickbush e Eickbush, 2007).

Em suma, uma grande distribuição dispersa dos fragmentos RT dos retroelementos Ty1-*copia*-like e Ty3-*gypsy*-like foi notada ao longo dos cromossomos de *G. max*, *P. vulgaris*, *P. lunatus*, *V.*

unguiculata e *V.radiata*, a qual deve ser atribuída à alta taxa de mutação e à baixa pressão seletiva, associadas ao mecanismo de retrotransposição ocorrentes de forma independente ao longo da diferenciação cariotípica de tais espécies. Por outro lado, considerando que as mutações originam distintas famílias de retroelementos, as quais se diferenciam pelas suas atividades de inserção em determinadas regiões cromossômicas, ressalta-se que o acúmulo dos elementos transponíveis pode ocorrer em regiões preferenciais, como heterocromatina e loci de DNAr, refletindo uma distribuição diferenciada de Ty1-*copia*-like e Ty3-*gypsy*-like nos genomas das leguminosas estudadas.

Agradecimentos

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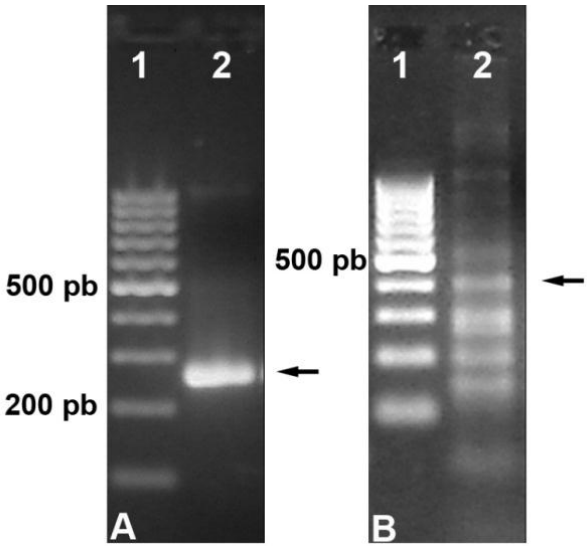

Figura 1. Amplificação por PCR do domínio RT do Ty1-*copia*-like (A) e Ty3-*gypsy*-like (B), a partir do DNA genômico de *Vigna unguiculata*, utilizando *primers* degenerados. As linhas 1 correspondem ao marcador de peso molecular 100 pb (Fermentas), enquanto que, as linhas 2 representam o produto da PCR para os respectivos domínios RT. Setas indicam os fragmentos que foram isolados e purificados.

A) *Vu*03 clone sequence Ty1-*copia*-like

```
1...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...73
GAGAATTCACCGCGTTTTTGCACGGTAATTTGGAGGAAGAGATATACATGAAGCAACCTGATGGTTTTCTTGT
TAAAGGCAAGGAAGATATGTGTGTAGACTAAGGAAGAGTCTATACGGTTTGAAGCAGGCTCCAAGGCAGTGGT
ATAAGAAGTTTGAGTCTTTTATGTGTGAGCAGGGTTACATGAAGACTACTTCTGATCATTGTGTCTTTGTTAA
AAGTTTTTCTAATGATGACTTTATTATCCTGTTATTGTACGTAGATGACATGGATCCTCA
...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...279
```

B) Ty1-*copia*

Access	Species	Sequence	Score	E-value	Identity
	V. unguiculata	GKEEYVCRLRKSLYGLKQAPRQWYKKFESFMCEQGYMKTTSDHCVF DFIILLLYVDDM			
Y12763.1	*V. unguiculata*	GKEDYVCRLRKSLYGLKQAPRKWYKKFESFMCEQGYKKTTSDHCVF DFIILLLYVDDM			
AAT90460.1	*V. radiata*	GKEDYVCRLRKSLYGLKQAPRQWYKKFESVMCEQGYKKTTSDHCVF DFIILLLYVDDM	161	2e-38	97%
CAJ09744.1	*C. sinensis*	GKEDYVCQLKKSLYGLKQAPRQWYKKFESVMEEQGYMKTISDHCVF DFIILLLYVDDM	151	3e-35	96%
CAA13065.1	*S. tuberosum*	GKENFVCKLKKSLYGLKQAPRQWYKKFESVMGEQGYKKTSSDHCVF DFIILLLYVDDM	144	3e-33	97%
ABD18968.1	*O. crenata*	GKEDYVCRLRKSLYGLKQAPRQWYKKFESVMGEQGYRKTTLDHCAF DFIILLLYVDDM	147	4e-34	96%

C) *Vu*02 clone sequence Ty3-*gypsy*-like

```
1...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...73
AGATGTGCGTGGATTATAGGCAGCTAAACAAATTAACAATTAAAAATCGGTATCCGTTACCGAGGATTGACGA
TCTACTAGATCGGTTGAAAGGGGCAGGAATATTCTCTAAGATAGACCTGCGATCTGGATATCATCAGATCTTG
GTGAAGCCGGAGGATGTACAAAAGACGGCGTTCAGGTCGAGGTATGGCCACTACGAATATGTAGTGATGCCAT
TCGGAGTCACGAATGCACCAGCCATATTCATGGATTACATGAATAGAATCTTTCGGCCGTGGTTAGATAAGTT
TGTTGTCGTTTTCATAGATGACATACTTATTTACTCGAAGACTAGGGAAGAGCATGTTGACCACTTGAGGGTA
GTATTGAAGATACTCAGGGATCATCAGTTGTATGCGAAACTCACCAAGTGTA
...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...417
```

D) Ty3-*gypsy*

Access	Species	Sequence	Score	E-value	Identity
	V. unguiculata	MCVDYRQLNKTIKNKYPLPRI DL FSKIDLRSGYHQ D RYGHYE VMPFGV YAKLTKC			
AAT85855.1	*V. radiata*	MCVDYRQLNKTIKNKYPLPRI DL FSKIDLRSGYHQ D RYGHYE VMPFGV YAKLSKC	253	5e-66	98%
ACU42190.1	*P. mume*	MCVDYRQLNRTIKNKYPLPRI DL FSKIDLRSGYHQ D RYGHYE VMPFGL YAKLNKC	233	5e-60	96%
ABD43107.1	*O. crenata*	MCVDYRELNRTIKNKYPLPRI DL FSKIDLRSGYHQ D RYGHYE VMPFGL YAKFNKC	224	3e-57	98%
ABD43161.1	*P. tunetana*	MCVDYRELNRTVKNKYPLPRI DL FSKIDLRSGYHQ D RYGHYE VMPFGL YAKFSKC	225	2e-57	98%
CAD45567.1	*E. guineensis*	MCVDYRELNKTIKNKYPLPRI DL FSKIDLRSGYHQ D RYGHYE VMPFGL FAKLTKC	223	9e-57	100%

Figura 2. Alinhamento e comparação dos clones dos retrotransposons com sequências depositadas no NCBI utilizando a ferramenta BLASTp. **A.** Sequência de nucleotídeo do retrotransposon Ty1-*copia*-like *de Vigna unguicuata*. **B.** Alinhamento da sequência do clone Ty1-*copia*-like de *V. unguiculta* com *V. radiata*, *Camellia sinensis*, *Solanum tuberosum* e *Orobanche crenata*. **C.** Sequência de nucleotídeo do retrotransposon Ty3-*gypsy*-like de *V. unguicuata*. **D.** Alinhamento da sequência do clone Ty3-*gypsy*-like de *V. unguiculta* com *V. radiata*, *Prunus mume*, *Orobanche crenata*, *Phelipanche tunetana* e *Elaeis guineesis*. Em **C** e **D**, os marcadores cinzas indicam os domínios conservados, enquanto que osos pretos indicam os aminoácidos conservados.

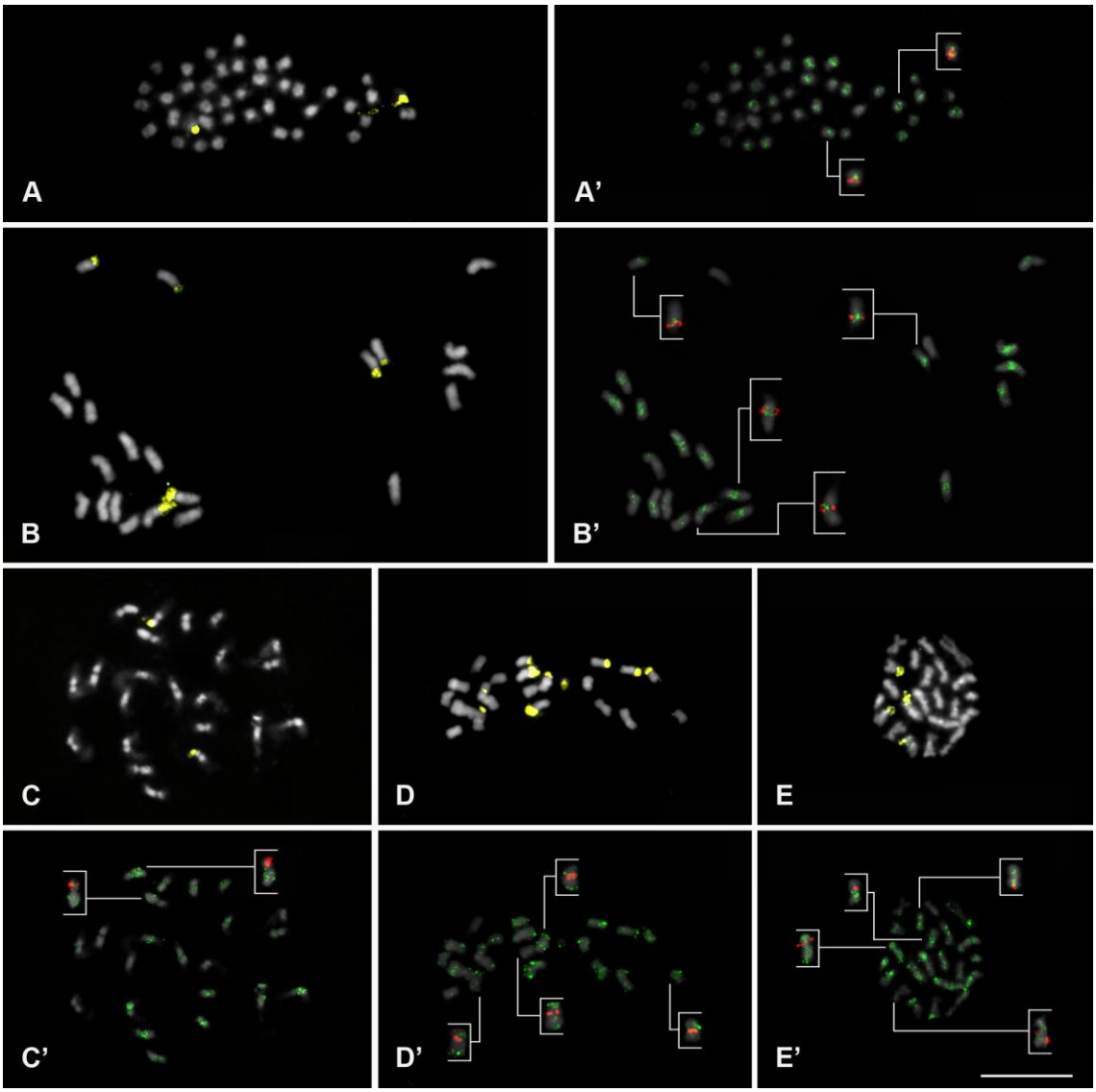

Figura 3. Células metafásicas de *Glycine max* (A-A'), *Phaseolus vulgaris* (B-B'), *P. lunatus* (C-C') *Vigna unguiculata* (D-D') e *V. radiata* (E-E') mostrando a distribuição dos sítios da sequência RT do retrotransposon Ty1-*copia*-like (em verde) e DNAr 45S (pseudocoloridos em amarelo). Os cromossomos foram contracorados com DAPI e pseudocoloridos em cinza (A, B, C, D e E). Inserções indicam os cromossomos portadores de DNAr 5S (A', B', C', D' e E'). Barra em E' = 5 µm.

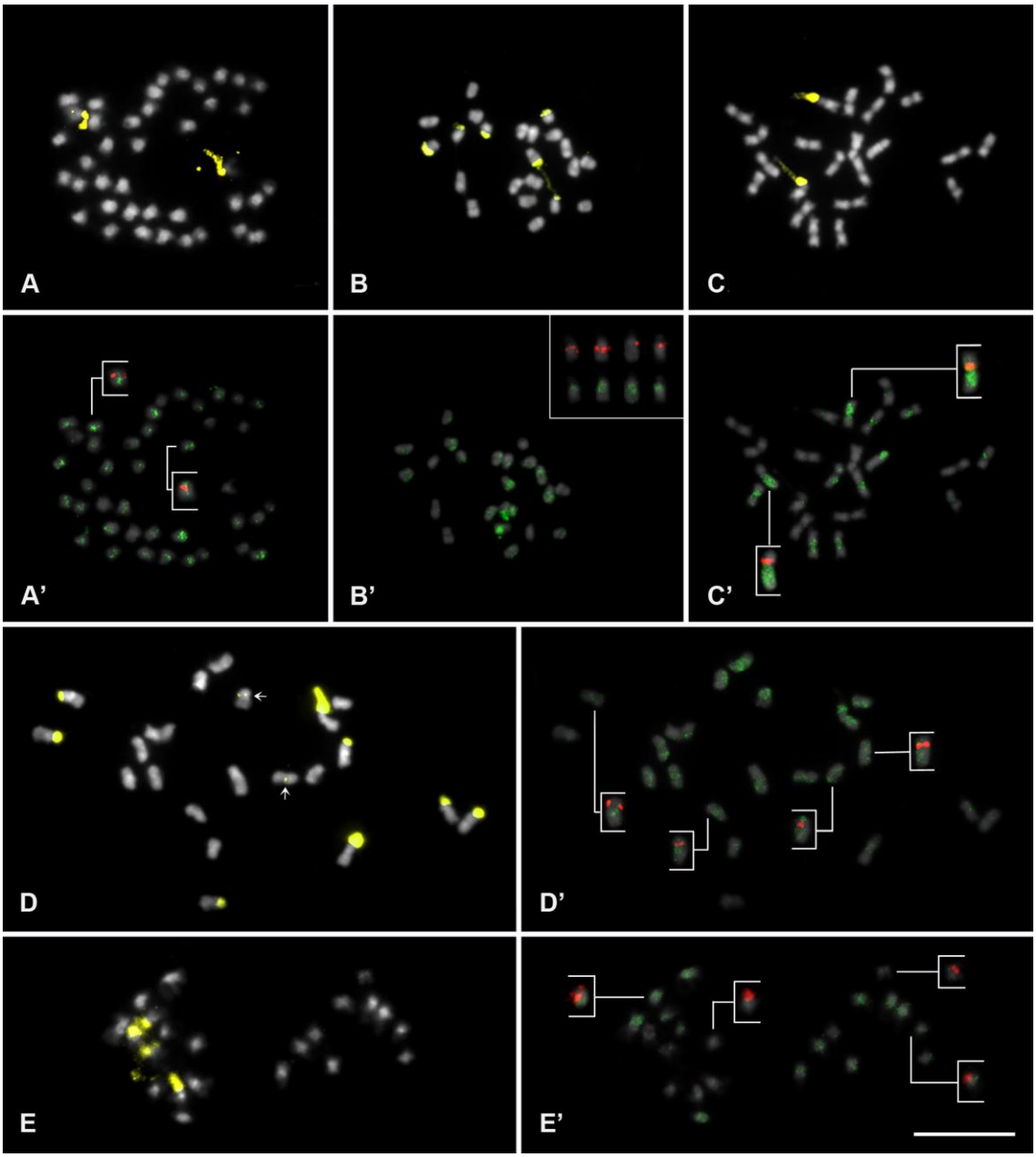

Figura 4. Células metafásicas de *Glycine max* (A-A'), *Phaseolus vulgaris* (B-B'), *P. lunatus* (C-C') *Vigna unguiculata* (D-D') e *V. radiata* (E-E') mostrando a distribuição dos sítios da sequência RT do retrotransposon Ty3-*gypsy*-like (em verde) e DNAr 45S (pseudocoloridos em amarelo). Os cromossomos foram contracorados com DAPI e pseudocoloridos em cinza (A, B, C, D e E). Inserções indicam os cromossomos portadores de DNAr 5S (A', B', C', D' e E'). Barra em E' = 5 µm.

Anexo I. Instrução para autores: Revista Open Plant Science Journal

The Open Plant Science Journal
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Keywords
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List of abbreviations (if any)
Conflict of interest (if any)
Acknowledgements (if any)
References
Appendices
Figures/illustrations (if any)
Chemical structures (if any)
Tables (if any)
Supportive/supplementary material (if any)

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Authors should also provide a short 'running title'.

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http://www.arabidopsis.org/portals/nomenclature/guidelines.jsp (*Arabidopsis*)
http://www.maizegdb.org/maize_nomenclature.php (Maize)

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Conference Paper:[5] Vanclay F. Agriculture, environment and society: Contemporary issues for Australia. In: Lawrence G, Vanclay F, Furze B, Eds. Macmillan, Melbourne. 1992; pp. 94-121.

Conference Proceedings: [6] Goss K, Chisholm T, Graetz D, Noble I, Barson M. Australian Agronomy Conferences. Proc. 5th (Perth), 6th (Armidale) and 7th (Adelaide) Conferences, 1989, 1991 and 1993. Sustaining the agricultural resource base. Office of the Chief Scientist, Dept. of the Prime Minister and Cabinet, AGPS Canberra. 1995.

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Volume with Part:[11] Abend SM, Kulish N. The psychoanalytic method from an epistemological viewpoint. Int J Psychoanal 2002; 83(Pt 2): 491 5.

Issue with Part: [12] Ahrar K, Madoff DC, Gupta S, Wallace MJ, Price RE, Wright KC. Development of a large animal model for lung tumors. J Vasc Interv Radiol 2002; 13(9 Pt 1): 923-8.

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*Journal abbreviations should follow the *Index Medicus*/MEDLINE.

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Anexo II. Instrução para autores: Revista Molecular Breeding

Molecular Breeding

New Strategies in Plant Improvement

Editor-in-Chief: Paul Christou

ISSN: 1380-3743 (print version)
ISSN: 1572-9788 (electronic version)

Journal no. 11032

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- Journal article

Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur J Appl Physiol 105:731-738. doi: 10.1007/s00421-008-0955-8

Ideally, the names of all authors should be provided, but the usage of "et al" in long author lists will also be accepted:

Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329

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Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. doi:10.1007/s001090000086

Book

South J, Blass B (2001) The future of modern genomics. Blackwell, London

Book chapter

Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

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Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

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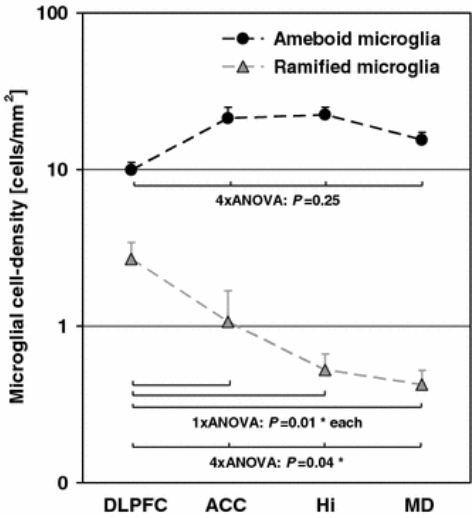

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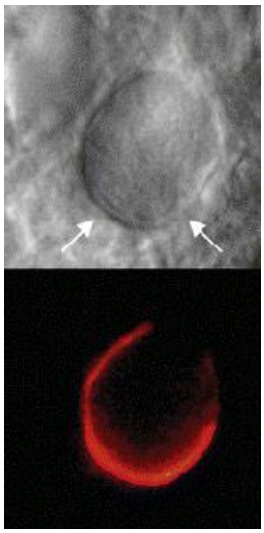

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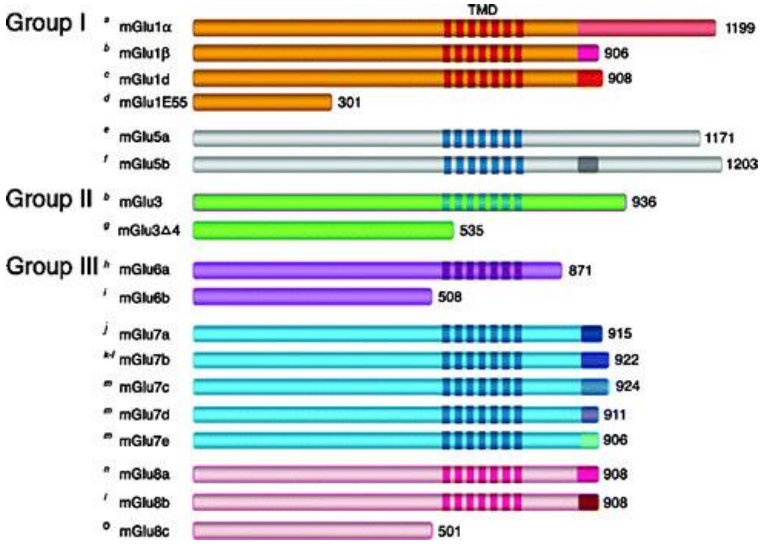

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Figure Lettering

- To add lettering, it is best to use Helvetica or Arial (sans serif fonts).
- Keep lettering consistently sized throughout your final-sized artwork, usually about 2–3 mm (8–12 pt).
- Variance of type size within an illustration should be minimal, e.g., do not use 8-pt type on an axis and 20-pt type for the axis label.
- Avoid effects such as shading, outline letters, etc.
- Do not include titles or captions within your illustrations.

Figure Numbering

- All figures are to be numbered using Arabic numerals.
- Figures should always be cited in text in consecutive numerical order.
- Figure parts should be denoted by lowercase letters (a, b, c, etc.).
- If an appendix appears in your article and it contains one or more figures, continue the consecutive numbering of the main text. Do not number the appendix figures, "A1, A2, A3, etc." Figures in online appendices (Electronic Supplementary Material) should, however, be numbered separately.

Figure Captions

- Each figure should have a concise caption describing accurately what the figure depicts. Include the captions in the text file of the manuscript, not in the figure file.
- Figure captions begin with the term Fig. in bold type, followed by the figure number, also in bold type.
- No punctuation is to be included after the number, nor is any punctuation to be placed at the end of the caption.
- Identify all elements found in the figure in the figure caption; and use boxes, circles, etc., as coordinate points in graphs.
- Identify previously published material by giving the original source in the form of a reference citation at the end of the figure caption.

Figure Placement and Size

- When preparing your figures, size figures to fit in the column width.
- For most journals the figures should be 39 mm, 84 mm, 129 mm, or 174 mm wide and not higher than 234 mm.
- For books and book-sized journals, the figures should be 80 mm or 122 mm wide and not higher than 198 mm.

Permissions

If you include figures that have already been published elsewhere, you must obtain permission from the copyright owner(s) for both the print and online format. Please be aware that some publishers do not grant electronic rights for free and that Springer will not be able to refund any costs that may have occurred to receive these permissions. In such cases, material from other sources should be used.

Accessibility

In order to give people of all abilities and disabilities access to the content of your figures, please make sure that

- All figures have descriptive captions (blind users could then use a text-to-speech software or a text-to-Braille hardware)
- Patterns are used instead of or in addition to colors for conveying information (color-blind users would then be able to distinguish the visual elements)
- Any figure lettering has a contrast ratio of at least 4.5:1

Springer accepts electronic multimedia files (animations, movies, audio, etc.) and other supplementary files to be published online along with an article or a book chapter. This feature can add dimension to the author's article, as certain information cannot be printed or is more convenient in electronic form.

Submission

- Supply all supplementary material in standard file formats.
- Please include in each file the following information: article title, journal name, author names; affiliation and e-mail address of the corresponding author.
- To accommodate user downloads, please keep in mind that larger-sized files may require very long download times and that some users may experience other problems during downloading.

Audio, Video, and Animations

- Always use MPEG-1 (.mpg) format.

Text and Presentations

- Submit your material in PDF format; .doc or .ppt files are not suitable for long-term viability.
- A collection of figures may also be combined in a PDF file.

Spreadsheets

- Spreadsheets should be converted to PDF if no interaction with the data is intended.
- If the readers should be encouraged to make their own calculations, spreadsheets should be submitted as .xls files (MS Excel).

Specialized Formats

- Specialized format such as .pdb (chemical), .wrl (VRML), .nb (Mathematica notebook), and .tex can also be supplied.

Collecting Multiple Files

- It is possible to collect multiple files in a .zip or .gz file.

Numbering

- If supplying any supplementary material, the text must make specific mention of the material as a citation, similar to that of figures and tables.
- Refer to the supplementary files as “Online Resource”, e.g., "... as shown in the animation (Online Resource 3)", “... additional data are given in Online Resource 4”.
- Name the files consecutively, e.g. “ESM_3.mpg”, “ESM_4.pdf”.

Captions

- For each supplementary material, please supply a concise caption describing the content of the file.

Processing of supplementary files

- Electronic supplementary material will be published as received from the author without any conversion, editing, or reformatting.

Accessibility

In order to give people of all abilities and disabilities access to the content of your supplementary files, please make sure that

- The manuscript contains a descriptive caption for each supplementary material
- Video files do not contain anything that flashes more than three times per second (so that users prone to seizures caused by such effects are not put at risk)

Anexo III. Instrução para autores: Revista Genetics and Molecular Biology

Genetics and Molecular Biology (formerly named Revista Brasileira de Genética/Brazilian Journal of Genetics - ISSN 0100-8455) is published by the Sociedade Brasileira de Genética (Brazilian Society of Genetics).

The Journal considers contributions that present the results of original research in genetics, evolution and related scientific disciplines. Manuscripts presenting methods and applications only, without an analysis of genetic data, will not be considered.

Although **Genetics and Molecular Biology** is an official publication of the Brazilian Society of Genetics, contributors are not required to be members of the Society.

It is a fundamental condition that submitted manuscripts have not been and will not be published elsewhere. With the acceptance of a manuscript for publication, the publishers acquire full and exclusive copyright for all languages and countries.

Manuscripts considered in conformity with the scope of the journal, judged by the Editor in conjunction with the Editorial Board, are reviewed by the Associate Editor and two or more external reviewers. Acceptance by the Editor is based on the quality of the work as substantial contribution to the field and on the overall presentation of the manuscript.

Genetics and Molecular Biology follows and Open-Access policy. Articles are made available in full content at SciELO. Back issues dating to 1998 are available through this site.

Back issues of the earlier titles (Brazilian Journal of Genetics and Revista Brasileira de Genética) are hosted at the journal's own site:http://www.gmb.org.br

Articles published since 2009 are also indexed at PubMed Central, and there available as a full text version.

The official abbreviation for Genetics and Molecular Biology is **Genet. Mol. Biol.**

There is a publication charge for manuscripts once they are accepted. For price information, exemptions and waiver policies, please consult the journal homepage http://www.gmb.org.br.

1. Manuscripts must be submitted through a Scholar One submission platform hosted at: http://mc.manuscriptcentral.com/gmb

The cover letter should be addressed to:

- Editor-in-Chief, Genetics and Molecular Biology

2. For submission the following instructions must be observed:

a) The manuscript that must be submitted by the Corresponding Author. This is the person who will also check the page proofs, and arranges for the payment that may incur during the editorial process.

b) Entering the following metadata is required: (i) the manuscript title, (ii) a short running title (max. 35 characters), (iii) the Abstract, and (iv) up to five keywords. All these items must be exactly the same as those figuring in the first two pages of the manuscript file. Furthermore, a cover letter addressed to the Editor is required. It must be edited by the corresponding author inserted within the reserved data field

c) Statements are required informing that the data have not been published and are not under consideration elsewhere, and that all authors have approved the submission of the manuscript. Furthermore, possible conflicts of interest (e.g. due to funding, consultancies) must also be disclosed.

d) The names of all co-authors, including institutional affiliations and e-mail addresses must be entered, as contact information for the Editorial Office.

e) In the referee suggestions field, up to five reviewer names can be entered by the author(s); valid e-mail contact addresses for these are required, in case they are selected by the editor. These suggestions can be made separately as preferred and not-preferred reviewer(s).

f) Files must be uploaded separately and identified according to file types. The main text file must include references and, if applicaple, figure legends, which must be typed on a separate page following the References and Internet Resources sections. Each table, figure and element containing supplementary material must be saved and uploaded in a separate file. Formats for text and tables are Word or RTF in Windows platform. Figures should be in TIFF or JPEG formats (see detailed instructions in 3.1.h).

g) Manuscripts including photos or any other identifiable data of human subjects must be accompanied by a copy of the signed consent by the individual or his/her guardian.

Failure to adhere to these guidelines can delay the handling of your contribution and manuscripts may be returned before being reviewed.

Special attention should be given to the structuring of the manuscript and correct language usage. These are important factors in the smooth running of the editorial and peer-review process, and can result in faster publication.

3. Categories of Contribution
3.1. Research Articles

Manuscripts must be written in English in double-spaced, 12-point type throughout; marked with consecutive line and page numbers, beginning with the cover page.

The following elements must start on a new page and be ordered as they are listed below:

a) **The title page** must contain: a concise and informative title; the authors' names (first name at full length); the authors' institutional affiliation, including department, institution, city, state or province and country; different

affiliations indicated with superscript Arabic numbers; a short running title of about 35 characters, including spaces; up to five key words; the corresponding author's name, postal address, phone and fax numbers and email address.

b) **The Abstract** must be a single paragraph that does not exceed 200 words and summarizes the main results and conclusions of the study. It should not contain references.

c) **The text** must be as succinct as possible. Text citations: articles should be referred to by authors' surnames and date of publication; citations with two authors must include both names; in citations with three or more authors, name the first author and use et al. List two or more references in the same citation in chronological order, separated by semi-colons. When two or more works in a citation were published in the same year, list them alphabetically by the first author surname. For two or more works by the same author(s) in a citation, list them chronologically, with the years separated by commas. (Example: Freire-Maia et al., 1966a, 1966b, 2000). Only articles that are published or in press should be cited. In the case of personal communications or unpublished results, all contributors must be listed by initials and last name (et al. should not be used). Numbers: In the text, numbers nine or less must be written out except as part of a date, a fraction or decimal, a percentage, or a unit of measurement. Use Arabic numerals for numbers larger than nine. Binomial Names: Latin names of genera, species and infraspecific taxa must be printed in italics; names of orders and families should appear in the Title and also when first mentioned in the text. URLs for programs, data or other sources should be listed in the Internet Resources Section, immediately after the References Section, not in the text.

The text includes the following elements:

Introduction - Description of the background that led to the study.

Material (or Subjects) and Methods - Details relevant to the conduct of the study. Statistical methods should be explained at the end of this section.

Results - Undue repetition in text and tables should be avoided. Comment on significance of results is appropriate but broader discussion should be part of the Discussion section.

Discussion - The findings of the study should be placed in context of relevant published data. Ideas presented in other publications should not be discussed solely to make an exhaustive presentation.

Some manuscripts may require different formats appropriate to their content.

d) **The Acknowledgments** must be a single paragraph that immediately follows the discussion and includes references to grant support.

e) The References Section: references must be ordered alphabetically by the first author surname; references with the same first author should be ordered as follows: first, as single author in chronological order; next, with only one more co-author in alphabetical order by the second author; and finally followed by references with more than two co-authors, in chronological order, independent of the second author surnames. In references with more than 10 authors only the first ten should be listed, followed by et al. Use standard abbreviations for journal titles as suggested byNCBI (http://www.ncbi.nlm.nih.gov/journals/).

Only articles that are published or in press should be included in this section. Works submitted for publication but not yet accepted, personal communications and unpublished data must be cited within the text. "Personal communication" refers to individuals other than the authors of the manuscript being submitted; "unpublished data" refers to data produced by at least one of the authors of the manuscript being submitted. Works of restricted

circulation (e.g., theses not available in public databases, congress abstracts not published in regular journals or public databases) should not be listed in this section.

Sample journal article citation: Breuer ME and Pavan C (1955) Behaviour of polytene chromosomes of Rhynchosciara angelae at different stages of larval development. Chromosoma 7:371-386.

Yonenaga-Yassuda Y, Rodrigues MT and Pellegrino KCM (2005) Chromosomal banding patterns in the eyelid-less microteiid lizard radiation: The X1X1X2X2:X1X2Y sex chromosome system in Calyptommatus and the karyotypes of Psilophtalmus and Tretioscincus (Squamata, Gymnophthalmidae). Genet Mol Biol 28:700-709.

Sample book citation: Dobzhansky T (1951) Genetics and Origin of Species. 3rd edition. Columbia University Press, New York, 364 pp.

Sample chapter-in-book citation: Crawford DC and Howard-Peebles PN (2005) Fragile X: From cytogenetics to molecular genetics. In: Gersen SL and Keagle MB (eds) The Principles of Clinical Cytogenetics. 2nd edition. Humana Press, New Jersey, pp 495-513.

Sample electronic article citation: Gotzek D, Ross KG (2009) Current status of a model System: The gene Gp-9 and its association with social organization in fire ants. PLoS One 4:e7713.

f) **Internet Resources Section:** this section should contain a list of URLs referring to data presented in the text, software programs and other Internet resources used during data processing. Date of consultation must be stated.

Sample Internet resource citation: Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/OMIM (September 4, 2005)

LEM Software, http://dir.niehs.nih.gov/dirbb/weinbergfiles/hybrid_design.htm (September 4, 2005)

g) **Tables** must be in Word format prepared with table tool (do not use space bar or tabulator). A concise title should be provided above the table. Tables must be numbered consecutively in Arabic numerals. Each column must have a title in the box head. Footnotes typed directly below the table should be indicated in lowercase superscript numbers. Tables that are to appear in the printed version must be saved in Word format and not as figures, so that they can later be fitted during typesetting. Each table must be saved and uploaded as a separate file.

h) **Figures** must be numbered consecutively in Arabic numerals. Images should be in TIFF or JPEG format and provided in separate files. Identify each illustration by the first author name and the number of the respective figure. Figures in Word, PowerPoint or Excel format cannot be published. Only sequence data can be presented in Word format. Journal quality reproduction will require grayscale resolution yielding 300 dpi, color figures should be at 600 dpi. These resolutions refer to the output size of the file; if it is anticipated that images will be enlarged or reduced, the resolutions should be adjusted accordingly. Figures composed of several elements should be sent as a single panel, obeying the print size definitions of the journal (single or two columns width). Scanned figures should not be submitted. Color illustrations are accepted.

Each figure/panel must be saved and uploaded as a separate file. When uploading, identify each illustration by the first author name and the number of the respective figure.

Figure legends must be included in the main text file and should be typed on a new page that immediately follows the tables.

i) **Nomenclature** should adhere to current international standards.

j) **Sequences** may appear in text or in figure. DNA, RNA and protein sequences equal to or greater than 50 units must be entered into public databases and accession numbers must be provided upon acceptance of the article. Failure to do so will delay publication.

k) **Data access:** reference should be made to availability of detailed data and materials used for reported studies.

l) **Ethical issues:** reports of experiments on live vertebrates must include a statement that the institutional review board approved the work and the protocol number must be provided. For experiments involving human subjects, authors must also include a statement that informed consent was obtained from all subjects. If photos or any other identifiable data are included, a copy of the signed consent must accompany the manuscript.

m) Supplementary Material: Data that the authors consider of importance for completeness of a study, but which are too extensive to be included in the published version, can be submitted as Supplementary Material. This material will be made available together with the electronic version. In case a manuscript contains such material, it should be appropriately identified within the text file. Supplementary material in tables should be identified as Table S1, Table S2, etc., in case of figures, they should be named accordingly, Figure S1, Figure S2. In addition, a list of this material should be presented at the end of the manuscript text file, containing the following statement: Supplementary material - the following online material is available for this article:

- Table S1 < short title >
- Figure S1 - < short title >

This material is available as part of the online article from http://www.scielo.br/gmb

3.2 Short Communications

Short Communications present brief observations that do not warrant full-length articles. They should not be considered preliminary communications;

- should be 15 or fewer typed pages in double spaced 12-point type, including literature cited;
- should include an Abstract;
- but no further subdivision, with introduction, material and methods, results and discussion; all in a single section and without headers.
- up to four items (tables and/or figures) may be submitted; Note: The title page, abstract and reference section format is that of a full-length Research Article. For Supplementary Material see instructions in item 3.1.m

3.3 Letters to the Editor

Relate or respond to recent published items in the journal. Discussions of political, social and ethical issues of interest to geneticists are also welcome in this form.

3.4 Review Articles

Review Articles are welcome. The Editor should be contacted prior to submission.

3.5 Book Reviews

Publishers are invited to submit books on Genetics, Evolution and related disciplines, for review in the journal. Aspiring reviewers may propose writing a review.

3.6 History, Story and Memories

Accounts on historical aspects of Genetics relating to Brazil.

4. Articles accepted for publication

Once an article is accepted, the Editorial Office will send it to a copy editor for language and technical corrections. If major corrections were proposed, the manuscript with the highlighted corrections will be returned to the corresponding author for approval. The final version approved by the authors must be free of any text/correction markings when returned to the Editorial Office.

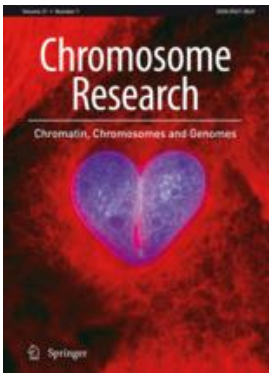
After typesetting, page proofs will be sent to the corresponding author. Changes made to page proofs, apart from typesetting errors, will be charged to the authors. Notes added in proof require Editorial approval.

A form of consent to publish and transfer of copyright will have to be signed by the corresponding author, also on behalf of any co-authors.

5. Reprints

Reprints are free of charge and provided as a pdf-file.

Anexo IV. Instrução para autores: Revista Chromosome Research



Style and Presentation
The manuscript should be typed with double spacing throughout, allowing for ample margins. The title page should show the paper title, names and addresses of all authors, a short running title, and fax and telephone numbers and the e−mail address for the corresponding author. The text of the paper should be arranged in the following sequence: Introduction, Materials and Methods, Results, Discussion, Acknowledgements and References. Informative legends should be provided for all illustrations and should be grouped together at the end of the paper, along with all tables. Subheadings may be inserted in the main text, but should not be numbered or lettered. Manuscripts should be written in clear, grammatical, idiomatic English. Spelling should conform to Webster's International Dictionary or The Concise Oxford English Dictionary and data should be presented simply and concisely, using Systeme International (SI) units.

Abbreviations
All abbreviations not obvious to the general reader should be defined the first time they are used. A complete alphabetically arranged list of all abbreviations used, including the definitions, should be included in the manuscript.

References
References should be cited in the text using the Harvard (name−date) system. Wherethere are three or more authors, only the first author's name should appear, followed by et al. Where several references are cited at the same point in the text, these should be arranged in chronological order. The reference list should be typed with double spacing and arranged in alphabetical order. References should include: names and initials of all authors (unless there are more than six authors, when only the first three authors should be given, followed by et al.); year of publication; full title of the article; source using abbreviations for journals as shown in Index Medicus; volume number; and first and last page numbers. Abstracts should be identified as such. For citations from books, the chapter title should be followed by the names and initials of all editors, the title of the book, edition, place of publication, publisher and first and last page numbers.

Examples:
Thomas HM, Harper JA, Morgan WG (2001) Gross chromosome rearrangements are occurring in an accession of the grass Lolium rigidum. Chromosome Res 9: 585−590.
Ohno S (2001) The one−to−four rule and paralogues of sex determining genes. In: Scherer G, Schmid M, eds. Genes and Mechanisms in Vertebrate Sex Determination. Birkhauser Verlag, pp 1−10.
Engel E, Antonarakis SE (2002) Genomic Imprinting and Uniparental Disomy in Medicine. New York: Wiley−Liss.

Only accepted papers should be referenced; all other material should be referred to in the text as 'in preparation', 'personal communication' 'unpublished observations' and should not be included in the reference list.

Citing Internet References
World Wide Web: All references should include the same information that would be provided for a printed source (or as much of that information as possible). The Web information is then placed at the end of the reference. It is important to use "Retrieved from" and the date because documents on the Web may change in content, move, or be removed from a site altogether. To cite a Web site in text (but not a specific document), it is sufficient to give the address (e.g., http://www.apa.org) there and no reference entry is needed. However, when citing a particular web page a citation in the text (e.g. Gaten 2000) and an entry in the reference list will be required.

For example:
Gaten E. (2000) Internet references. Retrieved from
http://www.le.ac.uk/biology/teach/mod300/ecitations.html 19/9/2000

E−mail: E−mail communications from individuals should be cited as personal communications. The format in the text (personal communications are not cited in the reference list) is as follows: (E. Gaten personal communication, March 28, 2001).
It is possible to send an e−mail note disguised as someone else. Authors − not journal editors or copy editors − are responsible for the accuracy of all references, which includes verifying the source of e−mail communications before citing them as personal communications in manuscripts.

One of the most comprehensive guides to citing internet references is provided by the American Psychological Association: http://www.apastyle.org/elecref.html

Keywords
Please provide 4 to 6 keywords which can be used for indexing purposes. Key words should be chosen carefully, they are essential to electronic search tools.

Tables and Illustrations
All tables and illustrations should be referred to in the text, with appropriate locations indicated in the text margin. Tables should present new information and not duplicate data included in the text. Every table should have a descriptive title and, if numerical measurements are given, the units should be included in the column heading. Line drawings should be supplied in a form suitable for high−quality reproduction. Axes should be labelled clearly; other lettering should be kept to a minimum. Avoid the use of fine tints, especially as background to text.

Electronic Figure Submission
Supply all figures electronically. For vector graphics, the preferred format is EPS; for halftones, please use TIFF format. MS Office files are also acceptable. Vector graphics containing fonts must have the fonts embedded in the files. Name your figure files with "Fig" and the figure number, e.g., Fig1.eps. Color illustrations should be submitted as RGB (8 bits per channel) TIFF. Photographs and photomicrographs should be cropped as close as possible to the area of interest and should be submitted as high resolution (>300dpi) as electronic files together with the manuscript. All photomicrographs must be accompanied by a scale bar with the equivalent measurement stated in the figure legend.

Colour figures
There are no charges for the publication of colour illustrations, either in the online or in the printed version of Chromosome Research.

Electronic Supplementary Material
Electronic Supplementary Material (ESM) for a paper will be published in the electronic edition of this journal provided the material is submitted in electronic form together with the manuscript and accepted after peer review. Electronic supplementary material will be published as received from the author without any conversion, editing, or reformatting. After acceptance for publication, ESM will be published as received from the author in the online version only. Reference will be given in the printed version.
ESM may consist of:
• Information that cannot be printed: animations, video clips, sound recordings (use PDF, QuickTime, .avi, .mpeg, animated GIFs, or any other common file format) Submit your text or presentation material in PDF format; .doc or .ppt files are not suitable for longterm storage.
• Information that is more convenient in electronic form: sequences, spectral data, etc.
• Large quantities of original data that relate to the paper, e.g. additional tables, large numbers of illustrations (colour and black & white), etc. Submission
• Supply all supplementary material in standard file formats.
• To accommodate user downloads, please keep in mind that larger-sized files may require very long download times and that some users may experience other problems during downloading.
Numbering If supplying any supplementary material, the text must make specific mention of the material as a citation, similar to that of figures and tables (e.g., ". . . as shown in supplement S3").
Legends
Legends must be brief, self-sufficient explanations of the ESM. ESM is to be numbered and referred to as S1, S2, etc.

Permission and Copyright
It is the responsibility of the author to obtain written permission for a quotation from unpublished material, or for all quotations in excess of 250 words in one extract or 500 words in total from any work still in copyright, and for the reprinting of figures or tables from unpublished or copyrighted material. Copyright in articles published in this journal is the property of Springer to the extent transferable. Authors are themselves responsible for obtaining permission to reproduce copyright material from other sources. There are no page charges or administration charges for papers published in this journal.

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In addition to the normal publication process (whereby an article is submitted to the journal and access to that article is granted to customers who have purchased a subscription), Springer now provides an alternative publishing option: Springer Open Choice™. A Springer Open Choice™ article receives all the benefits of a regular subscription-based article, but in addition is made available publicly (free of charge) through Springer's online platform SpringerLink. To publish via Springer Open Choice™, upon acceptance please visit www.springer.com/openchoice to complete the relevant order form and provide the required payment information. Payment must be received in full before publication or articles will publish as regular subscription-model articles. We regret that Springer Open Choice™ cannot be ordered for published articles. Please note that funding agencies with mandatory open access or public access (i.e. USA NIH and the UK Wellcome Trust) and several other institutes (The Max Planck in Germany, the Dutch UKB and others) have special arrangements for Open Access publishing with Springer. Please consult your funding agency's representative for specific details.

Proofs
Proofs will be sent to the corresponding author by email. Your response, with or without corrections, should be sent within 72 hours.
Off-prints
Authors may order additional off-prints on the form which accompanies the proofs.

Additional Information

Peter Butler
Publishing editor for Chromosome Research
Springer Science & Business Media
P.O. Box 17
3300 AA Dordrecht
The Netherlands
E-mail: Peter.Butler@springer.com

Anexo V. Súmula curricular

Lidiane Lindinalva Barbosa Amorim
Curriculum Vitae

Dados pessoais

Nome Lidiane Lindinalva Barbosa Amorim
Nascimento 29/01/1984 - Recife/PE - Brasil
CPF 046.170.314-99

Formação acadêmica/titulação

2009 Doutorado em Ciências Biológicas.
Universidade Federal de Pernambuco, UFPE, Recife, Brasil
Título: Análise genômica e transcriptômica em feijão-caupi: mapa genético e elementos transponíveis
Orientador: Ana Maria Benko Iseppon
Bolsista do(a): Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

2007 - 2009 Mestrado em Ciências Biológicas.
Universidade Federal de Pernambuco, UFPE, Recife, Brasil
Título: Construção de um mapa genético para feijão-caupi com marcadores moleculares ISSR, DAF e CAPS, Ano de obtenção: 2009
Orientador: Ana Maria Benko Iseppon
Bolsista do(a): Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

2011 - 2012 Graduação em Licenciatura em Ciências Biológicas.
Universidade Católica de Pernambuco, UNICAP, Recife, Brasil

2002 - 2006 Graduação em Bacharelado Em Ciencias Biológicas.
Universidade Federal Rural de Pernambuco, UFRPE, Recife, Brasil
Título: AVALIAÇÃO DE GENÓTIPOS DO FEIJÃO-CAUPI [Vigna unguiculata (L.) Walp.] PARA RESISTÊNCIA A ESTRESSE BIÓTICO E ABIÓTICO COM MARCADORES DAF (DNA Amplification Fingerprinting)
Orientador: Reginaldo de Carvalho - UFRPE; Ana Mª Benko Iseppon - UFPE
Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico

---

Formação complementar

2010 - 2010 Curso de curta duração em Biossegurança de Organismos Geneticamente Modifica.
Universidade Federal do Ceará, UFC, Fortaleza, Brasil
Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico

2007 - 2007 Curso de curta duração em Aplicação da Biologia Molecular na Biotecnologia.
Universidade Federal de Pernambuco, UFPE, Recife, Brasil

2006 - 2006 Curso de curta duração em Bioinformática.
Faculdade de Medicina de Ribeirão Preto, USP, Brasil

2006 - 2006 Curso de curta duração em Contextualizando a genética.
Sociedade Brasileira de Genética- XVII Encontro de Genética do Nordeste, SBG, Brasil

2006 - 2006 Curso de curta duração em Indução de Mutação em Plantas.
Sociedade Brasileira de Genética- XVII Encontro de Genética do Nordeste, SBG, Brasil

2006 - 2006 Curso de curta duração em Biologia Molecular Aplicada a Virologia.
Faculdade de Medicina de Ribeirão Preto, USP, Brasil

2006 - 2006 Curso de curta duração em III Curso de Verão em Biologia celular e Molecular.
Faculdade de Medicina de Ribeirão Preto, USP, Brasil

2006 - 2006 Curso de curta duração em Curso de Extensão em Técnicas de Biologia Molecula.
Fundação de Apoio ao Desenvolvimento da Universidade Federal de Pernambuco, FADE/UFPE, Recife, Brasil

2006 - 2006 Curso de curta duração em Marcadores Moleculares no Melhoramento de Plantas.
Embrapa Meio-Norte (Teresina-PI), EMBRAPA, Brasil

2005 - 2005 Curso de curta duração em Técnicas Moleculares, Bioinformática e Mapeamento.
Universidade Federal de Pernambuco, UFPE, Recife, Brasil

2004 - 2004 Curso de curta duração em Curso de Extensão "Técnicas de Hidroponia".
Universidade Federal Rural de Pernambuco, UFRPE, Recife, Brasil

2003 - 2003 Curso de curta duração em Capacitação de recurso humano Biossegurança de OGM.
Universidade Federal de Pernambuco, UFPE, Recife, Brasil

2002 - 2002 Curso de curta duração em Carcinicultura.
Confederação da Agricultura e Pecuária do Brasil, CNA, Brasília, Brasil

2002 - 2002 Curso de curta duração em Estudos Ecológicos em Ambientes Aquáticos.
Universidade Federal Rural de Pernambuco, UFRPE, Recife, Brasil

2002 - 2002 Curso de curta duração em Educação Ambiental.
Universidade Federal Rural de Pernambuco, UFRPE, Recife, Brasil

2002 - 2002 Curso de curta duração em Piscicultura.
Confederação da Agricultura e Pecuária do Brasil, CNA, Brasília, Brasil

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Atuação profissional

1. Universidade Federal de Pernambuco - UFPE

Vínculo institucional

2009 - Atual Vínculo: Bolsista , Enquadramento funcional: Doutorado , Carga horária: 40, Regime: Integral

2007 - 2012 Vínculo: Colaborador , Enquadramento funcional: Aluno Voluntário - Projeto de Extensão , Carga horária: 1, Regime: Parcial

2007 - 2009 Vínculo: Pesquisa e Desenvolvimento , Enquadramento funcional: Mestrando - Bolsista CAPES , Carga horária: 40, Regime: Integral

2005 - 2006 Vínculo: Colaborador , Enquadramento funcional: Estagiário - Bolsista PIBIC-CNPq-UFRPE , Carga horária: 20, Regime: Parcial

2004 - 2005 Vínculo: Colaborador , Enquadramento funcional: Estagiário - Bolsista PIBIC-

CNPq-UFRPE , Carga horária: 20, Regime: Parcial

Atividades

11/2007 - 12/2007 Outra atividade técnico-científica, Centro de Ciências Biológicas
Especificação:
participou das atividades da disciplina eletiva GENÉTICA NA AGRICULTURA, junto ao curso de BACHARELADO EM CIÊNCIAS BIOLÓGICAS (nível graduação), no decorrer do 2º SEMESTRE LETIVO DE 2007.

10/2007 - Atual Pesquisa e Desenvolvimento, Centro de Ciências Biológicas, Departamento de Genética
Linhas de pesquisa:
Gênomica Funcional , Melhoramento genético do Feijão-caupi (Vigna unguiculata) , Marcadores Moleculares em Vegetal , Biologia Molecular Vegetal , Mapeamento genético , Genes de Resistência a Patógenos em Feijão-Caupi , Bioinformática

07/2007 - 07/2012 Extensão Universitária, Centro de Ciências Biológicas
Especificação:
PARTICIPAÇÃO COMO ALUNO VOLUNTÁRIO - melhoria do ensino público e privado, através da elaboração e disponibilidade de um material didático complementar ao ensino na área de genética e de biotecnologia

08/2006 - 08/2006 Outra atividade técnico-científica, Centro de Ciências Biológicas
Especificação:
MONITORA DA PARTE PRÁTICA DO CURSO "Genes, Genomas e Tecnologias Genômicas", Depto de Genética- CCB - 21 a 31 de agosto de 2006, carga horária 30h.

06/2006 - 10/2006 Outra atividade técnico-científica, Centro de Ciências Biológicas
Especificação:
participou das atividades da disciplina eletiva GENÉTICA NA AGRICULTURA, junto ao curso de BACHARELADO EM CIÊNCIAS BIOLÓGICAS (nível graduação), no decorrer do 1º SEMESTRE LETIVO DE 2006

04/2006 - 08/2006 Outra atividade técnico-científica, Centro de Ciências Biológicas
Especificação:
TREINAMENTO NA CONSTRUÇÃO DE BIBLIOTECA EST (FEIJÃO-CAUPI) - Atividades: 1. Protocolo de extração do RNA Total ; 2. Captura de RNAm ; 3. Síntese da primeira e segunda fita de cDNA; 4) Transformação; 5) Mini-prep; 6) Sequenciamento

08/2005 - 09/2005 Outra atividade técnico-científica, Centro de Ciências Biológicas
Especificação:
Monitora Curso prático-teórico: Técnicas Moleculares, Bioinformática e Mapeamento

2. Universidade Federal Rural de Pernambuco - UFRPE

Vínculo institucional

2007 - 2012 Vínculo: Pesquisador - Colaborador , Enquadramento funcional: Pesquisador ,

Carga horária: 10, Regime: Parcial

2003 - 2003 Vínculo: Estagiário , Enquadramento funcional: Bolsista PIBIC-CNPq/UFRPE , Carga horária: 20, Regime: Parcial

2001 - 2003 Vínculo: Estagiário , Enquadramento funcional: Estágiario , Carga horária: 20, Regime: Parcial

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Atividades

05/2007 - 07/2012 Pesquisa e Desenvolvimento, Departamento de Biologia
Linhas de pesquisa:
Genética, Melhoramento e Biotecnologia da Cana-de-açúcar

04/2003 - 08/2003 Estágio, Departamento de Biologia, Laboratótio de Micologia
Estágio:
Biodiversidade de espécies fúngicas em ambiente hospitalar pediátrico

08/2001 - 04/2003 Estágio, Departamento de Pesca, Laboratório de Ictiologia
Estágio:
Alimentação de Centropomidae, Taxonomia de peixes estuarinos

3. Escola Superior Agrícola Luiz de Queiroz - ESALQ

Vínculo institucional

2006 - 2006 Vínculo: Estágio Depto Fitopatologia , Enquadramento funcional: Pesquisador - Estágiária , Carga horária: 12, Regime: Parcial

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Atividades

01/2006 - 02/2006 Estágio, Laboratório de Genética Molecular - Depto Fitopatologia
Estágio:
Marcadores Moleculares e sequenciamento

4. Secretaria de Educação de Pernambuco - GRE-NORTE

Vínculo institucional

2012 - Atual Vínculo: Professor Temporário , Enquadramento funcional: Professor, Regime: Parcial

---

Linhas de pesquisa

1. Genética, Melhoramento e Biotecnologia da Cana-de-açúcar

2. Bioinformática

3. Biologia Molecular Vegetal

4. Genes de Resistência a Patógenos em Feijão-Caupi

5. Gênomica Funcional

6. Mapeamento genético

7. Marcadores Moleculares em Vegetal

8. Melhoramento genético do Feijão-caupi (Vigna unguiculata)

Producão

Produção bibliográfica
Artigos completos publicados em periódicos

1. Costa, Maria Lucília M. da, **AMORIM, L. L. B.**, ONOFRE, A. V. C., MELO, LJOT, OLIVEIRA, M. B. M., CARVALHO, R., BENKO-ISEPPON, A. M. Assessment of Genetic Diversity in Contrasting Sugarcane Varieties Using Inter-Simple Sequence Repeat (ISSR) Markers. American Journal of Plant Sciences. , v.02, p.425 - 432, 2011.

2. BENKO-ISEPPON, A. M., CAVALCANTI, N.M.S., BERLAMINO, L. C., BEZERRA-NETO, J. P., **AMORIM, L. L. B.**, FERREIRA NETO, J. R. C., PANDOLFI, V., AZEVEDO, H. M. A., SILVA, R. L. O., SANTOS, M.G., ALVES, M.V.S., KIDO, E. A. Prospecção de genes de resistência à seca e à salinidade em plantas nativas e cultivadas. Revista Brasileira de Geografia Física. , v.06, p.1112 - 1134, 2011.

Trabalhos publicados em anais de eventos (completo)

1. BERLAMINO, L. C., WANDERLEY, A.C., SOARES-CAVALCANTI, N. M., PANDOLFI, V., **AMORIM, L. L. B.**, BENKO-ISEPPON, A. M. Analysis and annotation of chickpea bacterial artificial chromosome sequences. In: 8th Computational Intelligence Methods for Bioinformatics and Biostatistics, 2011, Gargnano, Itália. **Annals of the 8th Computational Intelligence Methods for Bioinformatics and Biostatistics,**. , 2011. v.8. p.1 - 12

2. **AMORIM, L. L. B.**, BERLAMINO, L. C., PIRECK, B., SOARES-CAVALCANTI, N. M., WANDERLEY, A.C., PANDOLFI, V., BRASILEIRO-VIDAL, A.C., BENKO-ISEPPON, A. M. Bioinformatics analysis of class II transposable elements in the cowpea genome as compared with the actual soybean knowledge In: 8th Computational Intelligence Methods for Bioinformatics and Biostatistics, 2011, Gargnano, Itália. **Annals of the 8th Computational Intelligence Methods for Bioinformatics and Biostatistics,**. , 2011. v.8. p.1 - 12

3. COSTA, MLM, **AMORIM, L. L. B.**, MELO, LJOT, BENKO-ISEPPON, A. M., CARVALHO, R. Diversidade Genética em variedades de Cana-de-açúcar (Saccharum spp.) com uso de marcadores ISSR In: 9º Congresso Nacional STAB, 2008, Macéio. **9º Congresso Nacional STAB**. , 2008.

Trabalhos publicados em anais de eventos (resumo)

1. ARAÚJO, F.T., MATOS, M.K.S., ONOFRE, A. V. C., **AMORIM, L. L. B.**, PIERECK, B. Análise da transferibilidade e polimorfismo de locos microssatélites de Phaseolus vulgaris para Vigna unguiculata In: XIX Encontro de Genética do Nordeste (ENGENE), 2012, Petrolina -PE, Juazeiro - BA. **ENGENE - A genética, a natureza e o ser humano: mudando mentalidades e transformando vidas**. , 2012.

2. LIMA, M. O., SILVA, S., SANTOS, R. F., **AMORIM, L. L. B.**, BEZERRA-NETO, J. P., PANDOLFI, V., BENKO-ISEPPON, A. M. CHARACTERIZATION AND MACROSYNTENY ANALYSIS OF THE SOS PATHWAY IN CASTOR BEAN In: 8th International Conference of the Brazilian Association for Bioinformatics and Computational Biology – X-Meeting, 2012, Campinas, São Paulo. **8th International Conference of the Brazilian Association for Bioinformatics and Computational Biology – X-Meeting**. , 2012.

3. PIERECK, B., **AMORIM, L. L. B.**, BEZERRA-NETO, J. P., BERLAMINO, L. C., BENKO-ISEPPON, A. M. Identificação de domínios conservados de transposons em sequências de feijão-caupi (Vigna unguiculata) In: XIX Encontro de Genética do Nordeste (ENGENE), 2012, Petrolina -PE, Juazeiro - BA. E**NGENE - A genética, a natureza e o ser humano: mudando mentalidades e transformando vidas**. , 2012.

4. **AMORIM, L. L. B.**, LIMA, M. O., PIERECK, B., SILVA, S., BEZERRA-NETO, J. P., PANDOLFI, V., BENKO-ISEPPON, A. M. Key members for flavonoid biosynthesis in cowpea Vigna unguiculata (L.) Walp.]: abundance, diversity and in silico expression In: **Brasileiro de Genética**2012, , Foz do Iguaçu - PR.

5. MATOS, M.K.S., ARAÚJO, F.T., **AMORIM, L. L. B.**, ONOFRE, A. V. C., PIERECK, B., BENKO-ISEPPON, A. M. MARCADORES SSR POLIMÓRFICOS ENTRE PARENTAIS DO FEIJÃO-CAUPI CONTRASTANTES PARA A RESISTENCIA AO VIRUS DO MOSAICO SEVERO In: V Sicbio, 2012, Recife, Pernambuco. **V Sicbio - Do laboratório à sociedade:aprimorando ideias e melhorando a qualidade de vida**. , 2012.

6. FELIX, A. M. S., **AMORIM, L. L. B.**, BRASILEIRO-VIDAL, A.C., ARRIEL, N.H.C, BENKO-ISEPPON, A. M. Seleção de primers DAF e ISSR para análise de diversidade molecular de espécies do gênero Jatropha In: XIX Encontro de Genética do Nordeste (ENGENE), 2012, Petrolina -PE, Juazeiro - BA. **ENGENE - A genética, a natureza e o ser humano: mudando mentalidades e transformando vidas**, 2012.

7. ARAÚJO, F.T., MATOS, M.K.S., **AMORIM, L. L. B.**, ONOFRE, A. V. C., PIERECK, B., VASCONCELOS, S., BENKO-ISEPPON, A. M. TRIAGEM DE MARCADORES SSR POLIMÓRFICOS EM GENÓTIPOS SUPERIORES DO FEIJÃO-CAUPI VISANDO AO MAPEAMENTO GENÉTICO In: V Sicbio, 2012, Recife, Pernambuco.**V Sicbio - Do laboratório à sociedade:aprimorando ideias e melhorando a qualidade de vida**, 2012.

8. BEZERRA-NETO, J. P., **AMORIM, L. L. B.**, PANDOLFI, V., SANTOS, R. F., LIMA, J. P. P., SILVA, S., BERLAMINO, L. C., BENKO-ISEPPON, A. M. AN OVERALL EVALUATION OF THE EXPRESSED REACTIVE OXYGEN SPECIES (ROS) GENES IN COWPEA In: **X-meeting**, 2011, Florianópolis.

9. SANTANA, K. C. B., **AMORIM, L. L. B.**, PANDOLFI, V., BERLAMINO, L. C., GAZANNEO, L.R., KIDO, E. A., CROVELLA, S., GALDINO, S.L., BENKO-ISEPPON, A. M. Caracterização Defensina Putativa e Isolamento de seu Gene Codificante em Euphorbia hyssopifolia L.: Novas Perspectivas Terapêuticas In: **2º Encontro Brasileiro para Inovação Terapêutica**, 2011, Recife

10. BORTOLETI, K. C. A., BRASILEIRO-VIDAL, A.C., VASCONCELOS, EV, **AMORIM, L. L. B.**, PANDOLFI, V. DISTRIBUIÇÃO CROMOSSÔMICA DE RETROELEMENTO Ty3-gypsy-LIKE EM ESPÉCIES DE Glycine WILLD., Phaseolus L. E Vigna SAVI In: **III Simposio Latinoamericano de Citogenética y Evolución**, 2011, Corrientes.

11. PIERECK, B., BERLAMINO, L. C., **AMORIM, L. L. B.**, BENKO-ISEPPON, A. M., BRASILEIRO-VIDAL, A.C. Identificação e caracterização comparatativa de elementos transponíves da classe II no genoma de Vigna unguiculata L. Walp (Fabaceae) In: 62º Congresso Nacional de Botânica, 2011, Fortaleza, CE. **62º Congresso Nacional de Botânica "Botânica e Desenvolvimento Sustentável"**, 2011.

12. PIERECK, B., **AMORIM, L. L. B.**, BERLAMINO, L. C., BEZERRA-NETO, J. P., ONOFRE, A. V. C., BRASILEIRO-VIDAL, A.C., BENKO-ISEPPON, A. M. IDENTIFICATION OF MUTATOR-LIKE TRANSPOSABLE ELEMENTS FROM COWPEA EXPRESSED SEQUENCES In**: X-meeting**, 2011, Florianópolis.

13. FELIX, A. M. S., **AMORIM, L. L. B.**, BRASILEIRO-VIDAL, A.C., LUCENA, A.M.A., ARRIEL, N.H.C., BENKO-ISEPPON, A. M. MARCADORES DAF E ISSR SELECIONADOS PARA ANÁLISES DA DIVERSIDADE EM

Jatropha In: **XL Congreso Argentino de Genética**, 2011, Corrientes, Argentina.

14. ARAÚJO, F.T., MATOS, M.K.S., ONOFRE, A. V. C., **AMORIM, L. L. B.**, LEITE, N.G.A., BENKO-ISEPPON, A. M. Marcadores SSR na detecção de polimorfismo em feijão-caupi visando o mapeamento de genes de resistência à seca In: 62º Congresso Nacional de Botânica, 2011, Fortaleza, CE. **62º Congresso Nacional de Botânica "Botânica e Desenvolvimento Sustentável"**, 2011.

15. SILVA JUNIOR, M.A.G, **AMORIM, L. L. B.**, LIRA-NETO, A. C., PINANGE, D. S. B., ARAÚJO, M.F.L., ALVES, M., BENKO-ISEPPON, A. M. RELATIONSHIPS AMONG NEOTROPICAL Croton SPECIES REVEALED BY ITSL AND ITS4 SEQUENCES In: **XL Congreso Argentino de Genética**, 2011, Corrientes, Argentina.

16. ARAÚJO, F.T., MATOS, M.K.S., ONOFRE, A. V. C., **AMORIM, L. L. B.**, LEITE, N.G.A., BENKO-ISEPPON, A. M. Seleção de polimorfismo de locos microssatélites visando ao desenvolvimento de mapas genéticos em feijão-caupi In: 62º Congresso Nacional de Botânica, 2011, Fortaleza, CE. **62º Congresso Nacional de Botânica "Botânica e Desenvolvimento Sustentável"**, 2011.

17. **AMORIM, L. L. B.**, SANTANA, K. C. B., GAZANNEO, L.R., PANDOLFI, V., BERLAMINO, L. C., BEZERRA-NETO, J. P., CROVELLA, S., KIDO, E. A., BENKO-ISEPPON, A. M. SEQUENCE ANALYSIS AND HOMOLOGY MODELING OF THE FIRST DEFENSIN FROM Etlingera elatior In: X-meeting, 2011, Florianópolis. **X-meeting**. , 2011.

18. LIMA, M. O., PIERECK, B., **AMORIM, L. L. B.**, CABRAL, D.G.A., BERLAMINO, L. C., BENKO-ISEPPON, A. M. SNAKINS: IN SILICO EVIDENCES FROM THE SOYBEAN TRANSCRIPTOME In: **X-meeting**, 2011, Florianópolis.

19. MATOS, M.K.S., ARAÚJO, F.T., ONOFRE, A. V. C., **AMORIM, L. L. B.**, PIERECK, B., LEITE, N.G.A., BORBA, T.C., BRONDANI, R.P.V., BENKO-ISEPPON, A. M. Transferability of SSR markers for diversity analysis and genetic mapping in cowpea In: **57º Congresso Brasileiro de Genética**, 2011, Águas de Lindóia, SP.

20. COSTA, MLM, **AMORIM, L. L. B.**, BENKO-ISEPPON, A. M., MELO, LJOT, CARVALHO, R. ANÁLISE DO POLIMORFISMO MOLECULAR EM VARIEDADES DE CANA-DE-AÇÚCAR (SACCHARUM SPP.) POR MEIO DE MARCADORES MOLECULARES. In: **56º CONGRESSO BRASILEIRO DE GENÉTICA**, 2010, Águas de Lindóia.

21. ONOFRE, A. V. C., **AMORIM, L. L. B.**, LEITE, N.G.A., COSTA, A.F., PANDOLFI, V., BENKO-ISEPPON, A. M. ASSESSING GENETIC DIVERSITY IN Vigna unguiculata CULTIVARS USING GENOMIC, GSS-DERIVED AND EST-DERIVED MICROSATELLITES In: **IX Feria-Congreso Latinoamericano de Biotecnología y IV Congreso Argentino de Biotecnología - BIOLATINA** 2010, Buenos Aires.

22. BENKO-ISEPPON, A. M., KIDO, E. A., PANDOLFI, V., BARBOSA, P.K., MONTE, S. J. H., BRANDAO, R. M. S. S., CAVALCANTI, N.M.S., BARBOSA SILVA, A., CALSA-JUNIOR, T., ROCHA. M.M., WINTER, P., KAHL, G., HORRES, R., MOLINA, C., JUNGMANN, R., **AMORIM, L. L. B.**, ONOFRE, A. V. C., FERREIRA NETO, J. R. C., GRANJEIRO, T. B. Brazilian cowpea transcriptome project: over 20 million expressed sequence tags to analyze and breed salinity and virus resistance In: **III Congresso Brasileiro de Biotecnologia**, 2010, Fortaleza.

23. DANTAS, L.G., SOUZA, A.C.B., **AMORIM, L. L. B.**, BENKO-ISEPPON, A. M., PEDROSA-HARAND, A. Desenvolvimento de marcadores moleculares para análises populacionais em Podocarpus sellowii In: XVIII ENGENE – Encontro de Genética do Nordeste, 2010, Jequié.

24. SILVA, M.L., QUEIROZ, M.A., BENKO-ISEPPON, A. M., **AMORIM, L. L. B.**, SILVEIRA, L.M. DIVERGÊNCIA GENÉTICA ENTRE ESPÉCIES DE CITRULLUS In: **I Congresso Brasileiro de Recursos Genéticos**, 2010, Salvador.

25. **AMORIM, L. L. B.**, LEITE, N.G.A., ONOFRE, A. V. C., COSTA, A.F., Damasceno-Silva, K.J., ROCHA. M.M., FREIRE FILHO, F. R., KIDO, E. A., BENKO-ISEPPON, A. M. GENETIC DIVERSITY AMONG COWPEA CULTIVARS AS REVEALED BY ISSR AND DAF MARKERS In: **IX Feria-Congreso Latinoamericano de**

Biotecnología y IV Congreso Argentino de Biotecnología - BIOLATINA 2010, 2010, Buenos Aires.

26. BORTOLETI, KCA, BRASILEIRO-VIDAL, AC, **AMORIM, L. L. B.**, VASCONCELOS, EV, BENKO-ISEPPON, A. M.OCORRÊNCIA E DISTRIBUIÇÃO CROMOSSÔMICA DE RETROELEMENTO TY1-COPIA-LIKE EM ESPÉCIES DOS GÊNEROS VIGNA SAVI E PHASEOLUS L. MEDIANTE HIBRIDIZAÇÃO IN SITU FLUORESCENTE In: **56º CONGRESSO BRASILEIRO DE GENÉTICA**, 2010.

27. **AMORIM, L. L. B.**, OLIVEIRA, A. M., LIRA-NETO, A. C., PINANGE, D. S. B., CRUZ, G. A. S., CARVALHO, R., BENKO-ISEPPON, A. M. OTIMIZAÇÃO DA AMPLIFICAÇÃO DE REGIÕES INTERGÊNICAS NO GÊNERO CROTON L. (EUPHORBIACEAE) In: **XXXIII Reunião Nordestina de Botânica**, 2010, Aracaju.

28. **AMORIM, L. L. B.**, ONOFRE, A. V. C., CARVALHO, R., MORETZSOHN, M. C., FERREIRA NETO, J. R. C., SILVA, R. L. O., CORREIA, C. N., SILVA, M. D., SITTOLIN, I.M., ROCHA. M.M., ANDRADE, G.P, PIO-RIBEIRO, G., KIDO, E. A., BENKO-ISEPPON, A. M.PRELIMINARY GENETIC LINKAGE MAP OF COWPEA (Vigna unguiculata) In: la **IX Feria-Congreso Latinoamericano de Biotecnología y IV Congreso Argentino de Biotecnología - BIOLATINA** 2010, Buenos Aires.

29. FELIX, A. M. S., ONOFRE, A. V. C., **AMORIM, L. L. B.**, BENKO-ISEPPON, A. M. Preservação de amostras de Material Vegetal para Extração de DNA: Desenvolvimetno de uma Nova Metodologia In: I Congresso Brasileiro de Recursos Genéticos, 2010, Salvador. **Bancos de Germoplasma: descobrir a riqueza, garantir o futuro**, 2010.

30. ONOFRE, A. V. C., **AMORIM, L. L. B.**, LEITE, N.G.A., PANDOLFI, V., COSTA, A.F., BENKO-ISEPPON, A. M. RESISTANCE GENE ANALOG POLYMORPHISM (RGAP) FOR GENETIC MAPPING OF Vigna unguiculata In**: IX Feria-Congreso Latinoamericano de Biotecnología y IV Congreso Argentino de Biotecnología - BIOLATINA** 2010, Buenos Aires.

31. BRASILEIRO-VIDAL, A.C., KIDO, E. A., CALSA-JUNIOR, T., CARVALHO, M, SANTOS, N., ONOFRE, A. V. C., LEIMING, C. J. C., RAMOS, H. V., **AMORIM, L. L. B.**, SILVA, E. B. C., VASCONCELOS, S., LIRA-NETO, A. C., OLIVEIRA, A. R. S., SOUZA, A. A., SILVA, F. M. R., SILVA, S. D., BENKO-ISEPPON, A. M. A GENÉTICA E BIOTECNOLOGIA CHEGAM ÀS ESCOLAS In: **55º Congresso Brasileiro de Genética** - "Charles Darwin - A origem das Espécies: O livro que transformou a humanidade", 2009, Águas de Lindóia, SP.

32. ONOFRE, A. V. C., **AMORIM, L. L. B.**, PANDOLFI, V., AZEVEDO, H. M. A., CAVALCANTI FILHO, G. J. F., KIDO, E. A., SITTOLIN, I.M., ROCHA. M.M., FREIRE FILHO, F. R., ANDRADE, G.P, PIO-RIBEIRO, G., BENKO-ISEPPON, A. M. Análise de segregantes na identificação de marcadores ligados a genes de resistência a virose em feijão-caupi In: XII Congresso Brasileiro de Fisiologia Vegetal “Desafios para produção de alimentos e bioenergia”, 2009, Fortaleza, CE. **XII Congresso Brasileiro de Fisiologia Vegetal**. Sociedade Brasileira de Fisiologia Vegetal, 2009.

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