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POLYMORPHISM OF MICROSATELLITE MARKERS DEVELOPED FROM HOMOLOGOUS GENOMIC REGIONS OF *PHASEOLUS VULGARIS* TO *MELOIDOGYNE SSP.* RESISTANCE GENES IDENTIFIED IN SOYBEAN

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ABSTRACT

DNA markers can contribute to the genetic control selection of many diseases in plants. Nematodes belonging to genus Meloidogyne gather pathogens that cause damage and yield losses in *Phaseolus vulgaris*. Although the sources of genetic resistance variations are known in legumes such as soybeans, little is known about this feature in common bean crops. This study aimed to characterize the polymorphism of microsatellite markers developed to extend the resistance genes of *P. vulgaris* to *Meloidogyne* spp. Twenty-six cultivars were used to characterize nine microsatellite markers that have developed from homologous genomic regions of *P. vulgaris* to six genes identified in soybean (EREBP; Ext1; Ext2; Rhg1; Rhg4; Rmi1), which are associated with resistance to root-knot nematodes. Six of the nine microsatellite primers were polymorphic. In total, 12 alleles were produced - two alleles per amplified microsatellite locus. The mean gene diversity was 0.36; no sample presented heterozygosity and this outcome corroborates the fixation index equal to 1.00. Probability of identity and of exclusion multiloci were 0.01 and 0.78, respectively. Despite the reduced ability of microsatellite markers to detect polymorphism, they are the first DNA markers developed to extend the resistance genes of common beans to *Meloidogyne* ssp. available for validation studies.

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INTRODUCTION

Agricultural expansion is one of the main causes of changes in natural landscapes (Ferraz junior, 2004; MMA, 2018). Environmental changes due to planting procedures increasingly require improved control, management and production systems, due to the worsening and profile of new phyto sanitary issues (Xu, 2016). These changes led to the introduction of emerging pathogens and diseases that were not problem to many crop types in the past, but that became prominent at present times. Phytonematodes in common bean plants belonging to species *Phaseolus vulgaris* are an example of such pathogens. Nematodes are polyphage phytopathogens of broad geographic distribution that cause significant damage and losses in the field (Santini, 2014). Species belonging to genus *Meloidogyne* ssp. are particularly damaging to common bean crops, since they cause up to 90% yield loss (Abawi *et al*., 2005). This loss is justified by the fact that nematodes drain and capture the plant nutrients for their own development (Caromel and Gebhard, 2011). The biological control of these pathogens has the potential to be used in nematode management, since it is one of the main tools adopted to reduce the use of synthetic nematicides, which are expensive and highly toxic to humans and to the environment (Bettiol *et al*., 2014). Preventive actions, such as cleaning the machines and agricultural implements, and the use of healthy seedlings, are among other management measures adopted to control *Meloidogyne* ssp. (Briar *et al*., 2016). The crop rotation technique is not interesting to farmers, mainly because they are

forced to cultivate the land with unprofitable crops. Nematode control based on the selection of genotypes presenting genetic resistance has been promising for bean crops (Oliveira *et al*., 2018), as well as for other agricultural crops, such as sunflower (Dias *et al*., 2016), soybean (Teixeira *et al*., 2017) and cotton (Alves *et al*., 2017). Therefore, management practices used to control nematodes combined with genetically-resistant plants can mitigate the frequent use of chemicals, since they lead to sustainable agricultural practices. Carvalho *et al*. (2016) point out that nematode resistant plants effectively contribute to reduce losses in infested areas. Therefore, they bring along the possibility of increasing yield and reducing production costs, as well as of assuring greater competitiveness and sustainability. Although some publications have shown a source of variation in the genetic resistance to *Meloidogyne* spp. in legumes (Baida *et al*., 2011; Ndeve *et al*., 2018), the resistance of the common bean culture to this pathogen remains little explored. This gap in the knowledge about this topic highlights the importance of producing information and developing tools to help prospecting genes to improve genetic resistance to this pathogen. DNA markers are a biotechnological tool of fast assessment that helps outlining strategies for the use of the genetic resources available. Microsatellite markers, or simple sequence repeat (SSR), have been widely used to this end because of their wide genome distribution, hypervariability and ease polymorphism detection (Cardoso *et al*., 2013; Müller *et al*., 2014).

Markers in Expressed Sequence Tag (EST) have been prominent among SSR markers because of their strong bond to known function genes, high transferability between related species and low development cost (Zhang *et al*., 2014). In addition to the recent advances in total genomic sequencing, one finds considerable increase in the availability of ESTs in public databases, and this new scenario facilitates genetic homology studies with phylogenetically close species (Schmutz *et al*., 2014), such as *P. vulgaris* and *Glycine max*. Vieira *et al*. (2016) developed 26 candidate SSR markers for common beans from nematode resistance genes found in soybean; however they were not yet optimized on their PCR amplification conditions and characterized by their genetic information power. The present study advocates for the hypothesis that candidate SSR markers used to improve the resistance of common bean to nematodes present expressive polymorphic information power in common bean germplasm. Assumingly, the herein generated product can be used as preventive tool to identify polymorphism cases related to the phenotypic behavior of common beans due to the presence of *Meloidogyne* ssp. Thus, the aim of the present research was to characterize the polymorphic information content of candidate SSR markers in *P. vulgaris* associated with genes capable of enhancing the resistance of this plant to *Meloidogyne* ssp.

MATERIAL AND METHODS

Plant samples: All experiments and result analyses were carried out in the Laboratory of Molecular Genetics of Instituto Federal Goiano (IF Goiano), Urutaí Campus, Goiás State, Brazil. The 26 *Phaseolus vulgaris* accessions were obtained in partnership with the phytopathology laboratory of IF Goiano (Table 1). Three (3) seeds from each of the 26 common bean accessions were separately sown in plastic bags filled with previously-prepared soil at sand/clay ratio 2:1. Young leaves were collected with aluminum foil and ice, and stored at -20 ° C until gene extraction.

DNA extraction: Genomic DNA was obtained from young leaves of common bean plants by applying the 2% CTAB method proposed by (Doyle and Doyle, 1990), with modifications in the maceration mode. Leaf tissue samples of each accession were manually ground in separate in 2.0 ml tubes with a stainless steel sphere (6 mm) in liquid nitrogen (N2). The tubes were previously frozen in N2 and transferred to a capped rack; the leaf tissue sample was macerated under strong unidirectional (up and down) movements for 30 seconds. The extracted DNA was resuspended in 100 µl TE-RNaase solution (Tris HCL 1M pH=8.0, EDTA to 0.5M pH=8.0 and RNase to 10 mg/mL). Aliquots of extracted genomic DNA were quantified in 0.8% agarose gel electrophoresis and stained with ethidium bromide (0.5 µL/mL) through visual comparison of the fluorescence intensity of DNA bands with molecular weight known of λ phage DNA (50, 100 and 200 ng/µL). The genomic DNA was diluted to the final concentration and to 10 ng/µL.

Optimization of developed SSR markers: Twenty six primers pairs developed from heterologous *P. vulgaris* genomic regions to seven genes (EREBP; Ext1; Ext2; Rhg1; Rhg4; Rmi1 and Pectin Esterase 1) associated with resistance to root-knot nematode in soybean were tested to amplification for PCR (Vieira *et al*., 2016) (Table 2). Only two random genotypes were chosen for PCR optimization and amplification tests among the 26 cultivars of the germplasm characterized in the present study. PCR reactions were induced to the final volume 12 µl with 20 ng DNA, 1x buffer (50 mMKCl, 10 mM Tris-HCl pH 8.3 and 1.5 mMMgCl 2), 0.3 mM of each primer (foward and reverse), 0.25 mM of each dNTP and 1 U of Taq DNA polymerase. Reactions were carried out in thermocycler under the following temperature conditions and procedural steps: initial denaturation stage at 94 ° C for 5 minutes; 35 cycles of denaturation steps (1 minute at 94 ° C), annealing (1 minute, gradient test temperature) and extension (1 minute at 72 ° C) and, finally, final extension step at 72 °C for another 7 minutes. Twelve (12) annealing temperatures (AT) for each primer pair were stablished in the gradient test at ± 0.5 °C variation in the melting temperature (Tm) of each primer, based on the programming available in the GeneMate Series Advance thermocycler. The optimization temperaturestested for the primers at Tm = 55 °C were T1= 50°C, T2= 50.5°C, T3= 51.2°C, T4= 52.2°C, T5= 53.4°C, T6= 54.6°C, T7= 55.8°C, T8= 56.9°C, T9= 58.0°C, T10= 59.0°C, T11= 59.5°C, T12= 60.0°C. A set with 26 different accessions were grown in a bulk with five replicates, which was used in separate to feature the polymorphism of SSR markers. The bulk was used as the control to detect the heterogeneity of each accession. PCR conditions were equal to the ones described above, except for gradient AT, since the optimal AT of each primer was determined (Table 2).

The product of each PCR was separated through vertical electrophoresis in 4% polyacrylamide gel and stained with silver nitrate based on Creste *et al*. (2001). This product was analyzed under white light after gel development and drying. This process allowed identifying the genotypes and their quality in regard to band sharpness and to the existence, or not, of nonspecific amplifications. The sizes of amplified DNA fragments at each SSR locus were estimated through a standard molecular weight marker (Ladder 50 bp).

Data analysis: The amplified fragments of microsatellites or alleles of each *locus* were estimated in separate in the

Tablecurve 2D software, based on a better fit to the regression model, according to the migration distance (in cms) and standard size of DNA fragments obtained from the standard 50 bp marker. Genetic information descriptors of SSR *loci* were estimated in the GenAlEx 6.5 software (Peakall and Smouse, 2012) based on the following statistics: allele numbers in each amplified locus (NA), frequency of each allele per locus (fa), heterozygosity (HO), expected heterozygosity or gene diversity (HE), fixation index (F) and probability of identity (PI) and of exclusion (PE).

RESULTS AND DISCUSSION

In total,nine of the 26 pairs of microsatellite primers developed for *Phaseolus vulgaris* were genetically characterized in the present study, the others no generated PCR product (Table 1). Seven of these nine primers (IFRT02, IFRT03, IFRT06, IFRT15, IFRT25, IFRT26 and IFRT20) were satisfactorily optimized and amplified well-definedPCR product.

The primers IFRT09 and IFRT11 produced single amplification fragment greater than 1 kb, therefore, they were excluded from the experiment; IFRT20 was monomorphic and the others were polymorphic. All polymorphic primers are trinucleotides, except for the SSR loci IFRT09 and IFRT20, which are di-nucleotides. The herein evaluated microsatellite primer pairs stood out for having derived from DNA sequences of *P. vulgaris* presenting more than 70% homology in the DNA sequence of seven soybean genes (*Glycine max*). Soybean genes, such asRmi1 (Luzzi *et al*., 1994), Rhg1 and Rhg4 (Concibido *et al*., 2004), Extensin1, Extensin 2, Pectin Esterase 1 and EREBP (Pham *et al*., 2013) are described in the literature as capable of enhancing the genetic resistance to root-knot nematodes (Vieira *et al*., 2016) (Table 2).

The high transferability and homology between distinct species such as soybeans and common bean is possible because these species are phylogenetically close to each other, since SSR-EST transferability is higher than that of the genomic SSRs.

Table 1. Individuals used in the polymorphism analysis applied to the developed microsatellite primers

Cultivars	Institution	Grain Color	Cycle days	Growth	Origin	Genealogy
ANFC9	-	Light beige	94	Undetermined	Mesoamerican	-
Bayer	Bayer	Light beige and light brown stripes			Mesoamerican	-
BRSMG Uirapuru	IAPAR	Black	77	Undetermined type I	Mesoamerican	BAC29/PR1711/3NEP2/2/PUEBLA173/ICAPIJÃO
IPR Campos Gerais	IAPAR	Light beige and light brown stripes	88	Undetermined type II	Mesoamerican	-
Comercial All Tech	All tech	Beige and brown stripes	-	-	Mesoamerican	-
BRS Estilo	Embrapa	Beige and brown stripes	85	Undetermined type II	Mesoamerican	EMP 250 /4/ A 769 / A 429 / XAN 252 /V 8025 / PINTO VI 114
BRSMG UAI	Embrapa /UFLA/ EPAMIG /UFV	Beige and brown stripes	85	Undetermined type II	Mesoamerican	Obtainedbyrecurrentselectionfrom Carioca MG, CNFC 9454, CNFC 9455, CNFC 9458, CNFC 9466, CNFC 9471, CNFC 9484, IAPAR 81, 9876 LP and IPR Uirapuru.
PF	-	Beige and brown stripes	-	-	Mesoamerican	-
Master 1	-	Beige and brown stripes	-	-	Mesoamerican	-
Master 2	-		-		Mesoamerican	-
CIIO	-	Light beige and light brown stripes	-	-	Mesoamerican	-
DRK	-	Darkred	-	-	Mesoamerican	-
All Tech	Alltech	Beige and brown stripes	-	-	Mesoamerican	-
BRS FC402	Embrapa	Light beige and brown stripes	85	Undetermined type II	Mesoamerican	Crossing between enhanced lines LM 96200246 and LP 9632.
IAC Sintonia	IAC	Beige and brown stripes	-		Mesoamerican	-
BRS FC104	Embrapa	Light beige and light brown stripes	65	Undetermined type II	Mesoamerican	Crossing between lines CNFE 8009 and VC5.
Comercial Master	Embrapa	Beige and brown stripes	-	-	Mesoamerican	-
Perola	Embrapa	Light beige to gray and light brown stripes	90	Undetermined type II	Mesoamerican	-
IAC Tigre	IAC	Light beige and brown stripes	85	-	Mesoamerican	-
Madre Perola	Embrapa /UFV/UFLA	Light beige and light brown stripes	88	Undetermined type III	Mesoamerican	Crossing between lines AN 512666-0 and AN 730031.
Polaco	-	Light beige and light brown stripes			Mesoamerican	-
Jest	-	Beige and brown stripes			Mesoamerican	-
IAC Milênio	IAC	Light beige to gray and brown stripes	95	Undetermined type III	Mesoamerican	Gen 96A98 x (Pérola x Ouro Negro).
BRS Esteio	Embrapa	Black	85	Undetermined type III	Mesoamerican	-
IPR Tangara	IAPAR	Light beige to gray and brown stripes	87	Determined type II	Mesoamerican	Crossing between enhanced lines LP95-92 and IAPAR 31 x Pérola.
IPR Tuiuiu	IAPAR	Black	88	Undetermined type II	Mesoamerican	Crossing between enhanced lines LP96-72(IPRUirapuru) x Xamengo.

Table 2. Optimized annealing temperature and repeat motif of the nine candidate SSR primers bond to genes that provide resistance to *Meloidogyne* ssp. were selected for genetic polymorphism characterization in *P. vulgaris*

Primer	AT (° C)	ReplicationMotifs	Gene
IFRT02	50,5	(ATT)6	EREBP
IFRT03	59	(CTT)4	EREBP
IFRT06	52	(CGG)3(CTA)6(CCG)4	Ext1
IFRT09	56	(AT)5	Ext1
IFRT11	57	(TAC)9(CCA)3(CTA)3	Ext2
IFRT15	58	(ATT)5	Ext2
IFRT20	51	(TC)13	Rhg1
IFRT25	53,5	(GAA)4	Rhg4
IFRT26	59	(CGA)4	Rmi1

Table 3. Polymorphism descriptors calculated for six pairs of primers, which were developed for target regions of resistance to *Meloidogyne* ssp. in *Phaseulos vulgaris*. Allele numbers (Na), expected heterozygosity (He), observed heterozygosity (Ho) and fixation index (F), probability of identity (PI) and of exclusion (PE)

Locus	Na	Size	Fmáx	H_O	H_E	F	PI	PE
IFRT 03	2	389-419	0.95	0	0.10	1.00	0.82	0.08
IFRT 06	2	194-199	0.60	0	0.49	1.00	0.39	0.27
IFRT 26	2	348-358	0.58	0	0.50	1.00	0.38	0.28
IFRT 25	2	182-188	0.80	0	0.33	1.00	0.51	0.22
IFRT 02	2	266-270	0.42	0	0.42	1.00	0.43	0.25
IFRT 15	2	342-348	0.81	0	0.32	1.00	0.53	0.21
Mean	2	-	0.69	0	0.36	1.00	-	-
Total	12	-	-				0.01	0.78

This outcome corroborates results in the present study and is the indicative that SSR-EST is one of the useful resources of molecular markers in genomic studies (Grupta *et al*., 2003). The higher heterologous amplification rates can be attributed to the higher conserved DNA sequences belonging to the transcribed regions of genomes defined as EST - Expressed Sequence Tag. In total, 12 polymorphic alleles were produced from six SSR-ESTs, with two alleles per locus, on average, and base pairs ranging from 194-419 bp (Table 3). The maximum allele frequency exceeded 50% in all evaluated SSR-EST loci, except for IFRT02. This finding indicates uneven allele distribution and the prevalence of certain alleles in different cultivars. This high allele frequency reduced the informative power of certain loci, fact that pointed out mean gene diversity (HE) value 0.36, which ranged from 0.10 (locus IFRT03) to 0.50 (locus IFRT26). HO or observed heterozygosity value of all loci was equal to zero (0), and this result was in compliance with 100% inbreeding in the studied loci, which was indicated by Maximum Fixation Index (FIS) equal to 1.00. HO and FIS are estimates of genetic diversity, which is influenced by the reproductive system of the species or by the process to obtain the seeds from the cultivars. These seeds, such as those of *P. vulgaris*, are predominantly autogamous or self-polluting (Veloso, 2014). The discriminative power of the loci ranged from low to intermediate: POI value per locus = 0.38 (locus IFRT26) at 0.82 (locus IFRT03) and PE value = 0.08 (locus IFRT03) at 0.21 (locus IFRT15) (Table 3). Additionally, the multilocus values of PI and PE were 0.01 and 0.78, respectively. In other words, based on this PI value, the probability of selecting two random cultivars with the same genotype, by taking into consideration the assessed loci, is 1 in 100, whereas the PE value represents the likelihood of excluding non-gestational cultivars when the allelic incompatibility in the evaluated loci reaches 78%.

Although we expect low polymorphism level in locus EST-SSR of trinucleotide repeat motifs (Ellegren, 2004), as corroborated by Santana (2014) in studies with common bean; Muller *et al*. (2014) found opposite revealing SSR (tri-) loci with the highest polymorphic information content among 34 SSRs characterized from 88 Andean and Mesoamerican common bean cultivars. According to Chabane *et al*. (2005), the lowest EST polymorphism compared to its genomic counterpart resulted from the greatest conservation of DNA sequences in the transcribed regions, which can also produce higher fixation rates, since SSR-EST markers reduce the polymorphic character. On the other hand, Hanai *et al*. (2007) found great allelic amplitude in SSR-EST loci (2-12 alleles per locus) in the germplasm of 23 common bean cultivars (Andean and Mesoamerican) when they were compared to SSR-genomic loci (2-7 alleles per locus). Assumingly, the low amount of polymorphism in the herein developed SSR loci can be associated not only with the function of the transcribed region of the species' genome, but also with the genetic base of the adopted germplasm bank.

The observed low values per locus suggest genetic diversity reduction in common bean breeding programs due to genetic base narrowing in which cultivars and lineages are selected based on a limited number of genetically related parents. Genetic erosion evidence was observed in trials with advanced lineages conducted in Embrapa Arroz and Feijão VCU between 2003/2004 and 2011/2012: approximately 50% reduction in both genetic diversity (0.57 to 0.31) and number of alleles (7.8 to 4.1) in the carioca type, respectively (Cardoso *et al*., 2013). Moreover, there was high homogeneity in common bean cultivars among institutions of origin. This homogeneity can be attributed to the broad use of CIAT lines - bean cultivars derived from the different institutional breeding programs (Cardoso *et al*., 2014). However, it is important noticing that the herein developed and characterized SSR markers are available not only for genetic diversity studies, but also for associative validation studies on genes that enhance the resistance of common beans to *Meloidogyne* ssp. These markers can be considered a great advance for the common bean culture, since they are the first SSR markers developed to extend genes in order to enhance plant resistance to pathogens. Therefore, they can be used in molecular improvement programs, i.e., in selection assisted by DNA markers.

Conclusion

Finally, we can conclude that the transferability between different species, but relatively close, like soybean and common bean, allows to increase the efficiency in the transfer of genomic information through the homology of sequences of genes of interest, expanding the knowledge about functional DNA markers, and can then be used for breeding programs, such as selection assisted by DNA markers. Thus, with the present work six new microsatellite markers of *P. vulgaris* heterologous to target regions of genes that confer genetic resistance to nematodes in *Glycine max* are available for validation.

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