Identification of essentially derived soybean cultivars using microsatellite markers

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ABSTRACT - *Cultivars within a species are traditionally distinguished by morphological traits. In some species such as soybean however, varieties are generally obtained from very similar elite parent groups, which makes the morphological differentiation rather difficult. The aim of this study was to differentiate two soybean varieties by means of microsatellite markers. One variety was susceptible and the other resistant to soybean stem canker, the latter essentially derived from the former, in five backcross generations. The DNA used in the analysis was obtained from morphologically indistinguishable seed of the two varieties studied. Forty-two microsatellite loci distributed across the integrated genetic map of soybean were analyzed, of which one locus, Satt115, differentiated the two varieties, indicating that even essentially derived varieties can be discriminated by molecular markers.*

Key words: cultivar protection, *Glycine max*, molecular markers, stem canker.

INTRODUCTION

Soybean [*Glycine max* (L.) Merrill] is an annual herbaceous leguminous crop, with a high grain protein content and easy adaptation to diverse soil-climatic conditions. It is one of the main oil crops of the world, actually the most cultivated. This leguminous plant accounts for approximately 44% of the Brazilian agricultural production destined for exportation and is the crop that earns most foreign exchange for the country. In 2004, Brazil accounted for about 25% of the world's soybean production (Conab 2005).

In spite of the great number of commercial soybean varieties in Brazil, the genetic variability between them is minor, mainly because they were derived from the same set of some few ancestors (Hiromoto and Vello 1986, Abdelnoor et al. 1995).

Cultivars, within a species, are normally discriminated by morphological descriptors. In species with a narrow genetic base, as in the case of soybean, in which varieties are obtained by hybridization within an elite group of genetically similar parentals, novel varieties tend to be very similar and often indistinguishable by morphological traits (Lanza et al. 2000). The distinction becomes even more difficult when an essentially derived cultivar is differentiated from the one that originated it.

In Brazil, when the law of Cultivar Protection was backed by law no. 9.456 as of April 25, 1997 and regularized by decree no. 2.366 on November 05, 1997, a more precise identification of cultivars was needed. A system based on DNA markers that can identify a unique combination pattern of these markers for each variety

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is therefore essential to facilitate the protection of new varieties and ensure breeders' intellectual propriety rights.

The molecular identification of certain varieties, besides useful in the process of cultivar protection, represents a very powerful auxiliary tool in the analysis of genetic purity of seeds, and can be applied whenever the visual characterization leaves room for doubts (Lanza et al. 2000, Schuster et al. 2004).

Among the techniques of available markers, microsatellites are particularly interesting for variety identification. This technique is distinguished by simplicity, speed and accuracy in the generation of the genetic profiles, and is easily automated as well. Moreover, the characteristics of multiallelism and co-dominance make microsatellites, with their high content of genetic information per locus, an excellent marker in the development of unique genetic fingerprints in the discrimination of cultivars (Cregan et al. 1994, Thomas et al. 1994, Russel et al. 1997, Diwan and Cregan 1997). Several studies have described the application of these markers in the identification of cultivars. Rongwen et al. (1995) used 7 microsatellite primer pairs to characterize 96 soybean genotypes, and found between 11 and 26 alleles per locus. Only 4 primer pairs were sufficient to discriminate 95 of the 96 evaluated genotypes. Using 20 loci SSR, Diwan and Cregan (1997) were able to distinguish several soybean cultivars considered identical based on RFLPs, morphology and pigmentation characteristics. Brow-Guerrida et al. (2000) managed to identify groups of related soybean genotypes using only three microsatellite markers, instead of the 46 RAPDs markers used previously. With 12 microsatellite markers, Priolli et al. (2002) successfully distinguished a morphologically similar group of 186 soybean cultivars.

The purpose of this study was to distinguish cultivars Emgopa 313 and Jataí using microsatellite markers. Cultivar Jataí is essentially derived from Emgopa 313 and was obtained by means of five backcross generations.

MATERIAL AND METHODS

Plant material

Seed of the soybean varieties Emgopa 313 and Jataí was used. Jataí is resistant to soybean stem canker and was obtained through backcrossing with variety Emgopa 313 as recurrent parent, which is susceptible to the disease, and BR92-31910 as donor line, which is stem canker-resistant. The variety Jataí was essentially derived from Emgopa 313 (in five backcross generations), so the morphological descriptors of both are the same and the seeds are indistinguishable (Monteiro et al. 1999).

DNA Extraction

The DNA samples were obtained from a bulk of 10 seed of each variety. The seeds were ground in a mill and the DNA extracted according to a protocol proposed by McDonald et al. (1994), with slight modifications (Schuster et al. 2004).

Microsatellite amplification and fragment separation

A total of 42 SSRs primer pairs (Table 1), distributed across the integrated linkage map of soybean (Cregan et al. 1999) were used and synthesized by *Invitrogen Life Technologies* and *GibcoBRL*. The results of the previous analysis with about 100 pairs of microsatellite primers were used as selection criterion of the primers. The DNA of four soybean varieties, known to be divergent from each other, was used as standard in the analyses. Monomorphic primers, as well as those with problems of amplification, were discarded in the selection process.

The amplification reactions of microsatellites were performed in a total volume of 25 mL, containing PCR buffer (100 mmol.L^{-1} Tris-HCl and 500 mmol.L^{-1} KCl, pH 8.0), 20 mmol.L^{-1} MgCl$_2$, 2.5 µmol.L^{-1} of each of the deoxinucleotides (dATP, dTTP, dGTP and dCTP), 6 µ mol.L^{-1} of each primer pair, 1.0 unit Taq DNA Polymerase and 30 ng DNA template. The amplifications were performed in a Perkin Elmer thermal cycler (GeneAmp PCR System 9600) using the touch down program. This program consisted of a denaturation cycle at 94 °C during 4 min, an annealing step at 65 °C for 40s, followed by 10 touch-down cycles decreasing by 1 °C per cycle until reaching 55 °C, followed by 30 cycles at 55 °C for 40s each. In each cycle the respective temperatures of denaturation (94 °C/40s) and polymerization (72 °C/1 min) were maintained. The final step consisted of a polymerization cycle at 72 °C for 7 min. The amplified fragments were separated in 7% denaturing polyacrylamide gel containing formamide, visualized

Table 1. Primers used in the genetic characterization of the soybean cultivars Emgopa 313 and Jataí and the obtained alleles

Primer*	Linkage group (Cregan et al 1999)
Satt073	A1
Satt211	A1
Sat_115	A2
Satt177	A2
Sat_095	B1
Satt197	B1
Sat_009	B2
Satt294	C1
Satt100	C2
Satt316	C2
Satt357	C2
Satt077	D1a + Q
Satt184	D1a + Q
Satt157	D1b + W
Satt216	D1b + W
Sat_092	D2
Satt082	D2
Satt135	D2
Satt335	F
Satt115	G
Satt131	G
Satt191	G
Satt235	G
Satt275	G
Satt309	G
Satt353	H
Satt215	J
Satt287	J
Sat_044	K
Satt326	K
Satt337	K
Sat_113	L
Satt182	L
Satt238	L
Satt373	L
Satt527	L
Satt220	M
Satt551	M
Sat_033	N
Sat_108	O
Satt094	O
Satt129	Q

*The primer sequence and the nucleus of the repetitive sequence can be obtained from SOYBASE (http://129.186.26.94/SSR.html)

under ultraviolet light, after staining with ethidium bromide, and photographed using the Eagle Eye II imaging system. Fragment sizes were calculated using software One-Dscan (version 2.03 for windows).

Determination of similarity coefficients

The similarity coefficient was estimated based on the genealogy, considering that in each backcrossing generation, half of the genome of the parent donor is recovered. Consequently, in generation RC5, 98.4375% of the donor genome is expected to be recovered, which is the similarity coefficient, or coefficient of relatedness, between the essentially derived variety and its recurrent parent, after five generations of backcrossing.

For the molecular data, the similarity coefficient was obtained by the ratio of the number of identical alleles by the total number of alleles evaluated for the pair of varieties considered.

RESULTS AND DISCUSSION

Of the 42 microsatellite loci evaluated in the varieties Emgopa 313 and Jataí, only 43 alleles were obtained. In only one locus, Satt115, heteromorphism between the varieties was observed. This corresponds to a genetic similarity of 97.62% of the two cultivars. This high similarity index had been expected, since cultivar Jataí was essentially derived from Emgopa 313. Considering the genealogy of the varieties, after five backcross generations, the coefficient of relatedness (equivalent to the genetic similarity, as mentioned in Material and Methods) is 98.4375%, admitting that the donor parent in the backcrossing program (BR92-31910) was not related to Emgopa 313 (Monteiro et al. 1999).

In locus Satt115, an allele of 131pb of cultivar Emgopa 313 was found, as well as an allele of 149pb of cultivar Jataí (Figure 1). This locus thus characterizes these two cultivars, and is sufficient to distinguish them. This difference of 18pb between the amplified fragments permits that the cultivars are differentiated even in gels with lower resolution in the separation of amplified PCR fragments, as in the case of agarose gels.

Although microsatellite loci are very variable and an error in the DNA replication during gamete formation would result in the obtainment of new alleles, it is not to be expected that a new allele would surge and be fixed in a pure variety. A novel allele that could possibly
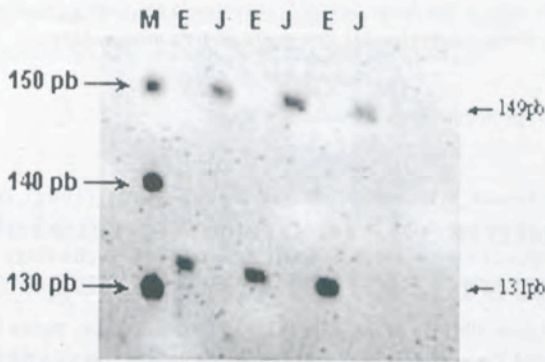
M E J E J E J

150 pb →

←149pb

140 pb →

130 pb →

←131pb

Figure 1. Polymorphism in the varieties Emgopa 313 (E) and Jataí (J) at locus Satt115. M = molecular weight marker (10 pb; Gibco)

surge by the described event would have an extremely low frequency in the variety, tending to disappear by genetic drift, unless it contributed with some adaptive advantage to the variety, as expected of microsatellite loci. The observation of this unique difference in the microsatellite locus is therefore sufficient to distinguish the two varieties with certainty.

Since Jataí is essentially derived from Emgopa 313, with a coefficient of relatedness of 98.4375%, based on genealogy, and 97.62% genetic similarity, based on the molecular analysis, and since Jataí is resistant to stem canker while Emgopa 313 is susceptible, it is possible that locus Satt115 is associated with the resistance gene in Jataí. This hypothesis can be tested by obtaining segregating populations for resistance to soybean stem canker.

A number of studies have demonstrated the efficiency of the use of molecular markers in distinguishing Brazilian soybean varieties (Abdelnoor et al. 1995, Priolli et al. 2002). These studies do not indicate if the coefficient of relatedness among any of the evaluated varieties is close to that between the

varieties Emgopa 313 and Jataí.

The coefficient of relatedness between these varieties (98.4375%) would indicate that among the loci that distinguish the parents used in the initial cross, one heteromorphic locus could be found for every 64 loci evaluated, i.e., 1.5625% genetic difference in the varieties. Since the other parent of Jataí (BR92-31910) was not available for this analysis, it is not possible to know how many of these loci would distinguish the parents. Nevertheless, one heteromorphic locus was identified by testing a smaller number of loci than would be expected by the coefficient of relatedness, indicating that the use of molecular markers can be useful in the characterization of cultivars, including essentially derived cultivars. However, the distinction of other essentially derived varieties may need the analysis of a larger number of loci to find the few existing differences between them.

CONCLUSION

Locus Satt115 was identified by the evaluation of 42 microsatellite loci distributed across the soybean linkage map. It distinguishes the varieties Emgopa 313 and Jataí, of which the latter is essentially derived from the first, with a coefficient of relatedness of 98.4375%. Our results allow the conclusion that microsatellite markers can be used for an efficient characterization of cultivars, including the essentially derived varieties.

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Identificação de cultivares essencialmente derivadas de soja com o uso de marcadores microssatélites

RESUMO - *A distinção de cultivares, dentro de uma espécie, é tradicionalmente feita por meio de caracteres morfológicos. No entanto, em algumas espécies, como a soja, as variedades são geralmente obtidas a partir de grupos elites parentais muito semelhantes, o que dificulta bastante a diferenciação morfológica. Neste trabalho, objetivou-se diferenciar duas variedades de soja por meio de marcadores microssatélites, sendo: uma suscetível e outra resistente ao cancro da haste da soja, sendo que a variedade resistente era essencialmente derivada da outra, com cinco gerações de retrocruzamentos. O DNA utilizado na análise foi obtido das sementes das duas variedades estudadas, morfologicamente indistinguíveis. Foram analisados 42*

locos de microssatélites distribuídos no mapa genético integrado da soja, e um loco, Satt115, distinguiu as duas variedades, indicando que mesmo variedades essencialmente derivadas podem ser caracterizadas por marcadores moleculares.

Palavras-chave: Marcadores moleculares, cancro da haste, *Glycine max*, proteção de cultivares.

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