Microsatellite markers linked to the locus of the watermelon fruit stripe pattern

R.N.C.S. Gama[1], C.A.F. Santos[2], R.C.S. Dias[2], J.C.S.F. Alves[3] and T.O. Nogueira[4]

[1]Programa de Pós-Graduação em Recursos Genéticos Vegetais, Universidade Estadual de Feira de Santana, Feira de Santana, BA, Brasil [2]Embrapa Semiárido, Petrolina, PE, Brasil
[3]Departamento de Tecnologia e Ciências Sociais, Universidade do Estado da Bahia, Juazeiro, BA, Brasil
[4]Departamento de Ciências Biológicas, Universidade de Pernambuco, Petrolina, PE, Brasil

Corresponding author: R.N.C.S. Gama
E-mail: renata.natalia@hotmail.com

ABSTRACT. Agronomic performance and external and internal appearance of watermelon (*Citrullus lanatus*) fruit are important traits that should be taken into consideration during the development of a new cultivar, as well as being the principal identification elements used by the consumer, which are based on the external appearance and quality of the fruit. Externally, the fruit can be characterized in terms of the shape, the color of the lower rind, and the presence of grooves and stripes, the stripes can be classified as clearly defined or diffuse.
The objective of this study was to identify microsatellite markers linked to the stripe pattern of watermelon fruit to support watermelon improvement programs, with the selection of this characteristic in the plantlet stage. F1 and F2 populations, result of a cross between the cultivars BRS Opara (clearly defined stripes) and Pérola (diffuse stripes), were phenotyped for their fruit stripe pattern. The CTAB 2X

protocol was used for DNA extraction and 116 microsatellite markers were examined in a group of F2 plants that had fruit with well-defined stripes and fruit with diffuse stripes. The microsatellite loci MCPI_05 and MCPI_16 exhibited a linkage to the stripe pattern at a distance of 1.5 and 1.8 cM, respectively, with LOD scores of 39.28 and 38.11, respectively, which were located on chromosome six of the watermelon genome. These markers can be used in marker-assisted selection in watermelon improvement programs, by various research institutions.

INTRODUCTION

The watermelon (*Citrullus lanatus*) is a member of the Cucurbitaceae, and is cultivated all over the world. There are various cultivars of this species, which differ from one another in terms of the external pattern of the fruit, the color of the pulp, the level of soluble solids, the yield, resistance to pests and diseases, and precocity, among other desirable agronomic traits. A summary of these morphological traits is provided by Dias et al. (2010) and Gama et al. (2013).

During the development of a new cultivar, it is necessary to consider the possible clients: producers, industry, and consumers (Borém and Miranda, 2013). According to Dias et al. (2010), the cultivar Crimson Sweet (and similar types), which is American in origin and has a clearly defined stripe pattern, is the most cultivated in Brazil, and accounts for over 90% of the consumer market. When selecting a cultivar for planting, the following should be taken into consideration: the fruit that is preferred by the consumer market, its tolerance of transport, the adaptation of the cultivar to the region, and its tolerance to disease and to physiological disorders. Therefore, genetic improvement programs should have various goals, including resistance to disease and pests, increased yield, and the external and internal appearance of the fruit, since appearance is the consumer's method of identifying the cultivar.

According to morphological descriptions of watermelons (Brazilian Ministry for Ag-riculture), the external pattern of watermelon fruit can be characterized in terms of the shape of the fruit, the color of the lower rind, the presence of grooves, the presence of stripes (well-defined or diffuse), the intensity of the stripe coloration, and the width of the stripes. The internal pattern can be characterized in terms of the thickness of the pericarp, coloration, and the firmness of the flesh (Brasil, 2009).

Classic improvement techniques for the selection of the watermelon fruit pattern can only be conducted during the fructification stage or during harvest, 40 and 80 days after sowing, respectively. Biotechnological techniques, such as microsatellite markers, make it possible to select individuals based on their genotype, independently of biotic or abiotic factors, and at any developmental stage of the plant. In this way, the identification of molecular markers that are linked to phenotypic traits, such as the stripe pattern of watermelon fruits, enables selection of the desired external pattern of the fruit still in the seedling stage, as well as a better understanding of the inheritance of the trait between progenies and a reduction in the number of individuals that have to be examined in the field (Bhering et al., 2009). Marker-assisted selection (MAS) tends to speed up improvement programs, because selection can be conducted during the initial stages of plant development, which is of fundamental importance for the development of new cultivars (Demore, 2008). Various molecular markers that are associated with different pheno-typic traits permit MAS, and can add various traits of interest to a new cultivar.

Microsatellite markers, also known as simple sequence repeats (SSRs), consist of units of approximately one to six nucleotides repeated in tandem (Oliveira et al., 2006; Caixeta et al., 2009). They have been developed for various cultivated plant species and are quickly replacing other marker types in various types of genetic studies, because of their reproduc-ibility and technical simplicity, the small amount of DNA that is required, their low cost, their great resolving power, and their high levels of polymorphism (Caixeta et al., 2009). Gama et al. (2013) used 36 molecular markers to characterize 17 watermelon cultivars, and observed the

formation of two groups, one that contained cultivars that exhibited a diffuse stripe pattern, and one that contained cultivars that exhibited a well-defined stripe pattern.

The authors also found that for the markers MCPI-05 and MCPI-16, all the cultivars that had well-defined stripe patterns had the same allelic pattern.

The objective of this study was to identify microsatellite markers linked to the stripe pattern of watermelon fruit to support watermelon improvement programs, with the selection of this characteristic in the plantlet stage.

MATERIAL AND METHODS

Plant material

Crosses were made by controlled hand-pollination (CHP), as described by Dias et al. (2001), between two watermelon cultivars that had different stripe patterns on their fruit: the cv. BRS Opara (well-defined stripes) and the cv. Pérola (diffuse stripes), to obtain the F1 population. Samples of young leaves were collected from each parent and stored in a freezer at -80°C until DNA extraction. After harvesting, the fruits from this cross were characterized, and the seeds were washed and dried. Seeds (F1) of one of these fruits were sown in polysty-rene trays and kept in a greenhouse for 12 days. At the beginning of seedling development, leaf samples of the F1 were collected for later DNA extraction and confirmation of hybridization by polymerase chain reaction (PCR) analysis, using microsatellite markers.

Seedlings of the F1 population were transplanted to the Experimental Field of Embrapa Semiárido, Petrolina, PE, Brazil. During the flowering stage, the autofecundation of these plants was conducted by CHP to obtain the F2 population. After harvesting, the fruits were characterized and the seeds were washed and dried.

A final field experiment was carried out, in which fruit from the parents of the F1 and F2 populations were characterized in terms of their stripe patterns (well-defined or diffuse stripes). One-hundred plants of the F1 population and 200 plants of the F2 population (the seeds used were collected from a single fruit), as well as thirty plants of each parent, were examined. During the initial seedling development stage, young and mature leaf samples were collected from the F2 population and stored in a freezer at -80°C until DNA extraction. During this stage, no leaf samples were collected from the parents or the F1 population, because this DNA had been extracted earlier.

DNA extraction and microsatellite analyses of the F1s

DNA extraction of the leaves was conducted using the Doyle and Doyle (1990) CTAB 2X protocol, modified to 7500 and 10,000 rpm at the first and the second centrifu-gations, respectively, and 2% beta- mercaptoethanol and incubation at 60°C for 30 min, for all the samples. The DNA was resuspended in a Tris-EDTA buffer and treated with RNAse to remove co-isolated RNAs. The quantification, and an integrity analysis, of the DNA were carried out on 0.8% agarose gel, and the samples were subsequently stored in a freezer at -20°C.

Five microsatellite markers were used (MCPI_04, MCPI_05, MCPI _11, MCPI_16, and MCPI_26) and were developed for the watermelon by Joobeur et al. (2006), for the inclusion of paternity and confirmation of the hybrids. These SSRs were selected based on the study by Gama et al. (2013), because they exhibited PCR products of different sizes for these cultivars. The PCR was conducted by following the protocol of Joobeur et al. (2004), with some modifications: the total volume of 12 µL contained 50 ng DNA, 5 µM of each primer, 0.1 mM dNTP, 1.5 mM $MgCl_2$, 1X PCR buffer, and 0.75 U Taq polymerase DNA enzyme. The programming of the thermocycler for the amplifications was as follows: an initial cycle of 94°C for 2 min, followed by 30 cycles at 94°C for 15 s, at 56°C for 30 s, and at 72ºC for 2 min, and a final cycle at 72°C for 30 min. Half of the volume from the reaction and 98% formamide denaturation buffer (10 mM EDTA, pH 8.0, 1 mg/mL xylene cyanol, and 1 mg/mL bromophenol blue) was added to the PCR solution, followed by complete de-naturation at 94°C for 5 min in a

thermocycler, then was immediately put on ice until use on a polyacrylamide gel. The amplification products were separated on 6% polyacrylamide gel, prepared on a glass plate of the sandwich type, with a capacity for sixty holes. A pre-run of 30 min at 35 W was conducted before adding the PCR samples. Two-and-a-half microliters of the denatured PCR on the 6% polyacrylamide gel was subjected to an electrophoresis cur-rent that was maintained for approximately 3 h, with a constant potential of 45 W. A 50-bp molecular-weight ladder (Fermentas) was charged at the lateral extremity of each gel. The gels were stained with silver nitrate, in accordance with the procedure described by Creste et al. (2001). To confirm that the obtained result of the cross between the cultivars was from

F1 plants, and was free of contamination, the hybrids were examined to ascertain whether they only exhibited the allelic patterns of the parents.

Phenotyping for the stripe patterns of watermelon fruit in the F1 and F2 populations and the parents

Harvesting was conducted manually, 75 days after sowing. The fruits were identified by the plant number, and taken to a barn where they were characterized in terms of morpho-logical descriptors for the watermelon. The number of fruits with well-defined or diffuse stripe patterns was recorded.

Genotyping the F2 population for stripe patterns on the fruit using microsatellite markers

A total of 116 microsatellite markers developed for the watermelon were analyzed, seven of which were developed by Jarret et al. (1996), 18 by Guerra-Sanz (2002), 36 by Joo-beur et al. (2006), and 55 by Ren et al. (2012), in order to select those primers that exhibited PCR products of different sizes among the parents. These were ten used to analyze five indi-vidual fruits with well-defined stripe patterns and five with diffuse stripe patterns from the F2 population, to identify alleles that were present in the individuals with well-defined stripe pat-terns and absent in the individuals with diffuse stripe patterns, and vice-versa. After verifying this characteristic with these thirty individuals, the selected primers were used to genotype the 200 individuals of the F2 population. The methodology used for DNA extraction, PCR, and use of microsatellite markers and revelation of the polyacrylamide gels was the same as was previously described for the F1 DNA extraction and microsatellite analyses.

Statistical analyses and identification of the chromosomal region of the stripe patterns of watermelon fruit

The data were annotated for the presence (1) or absence (0) of alleles. The data ob-tained by microsatellite markers and a visual evaluation of the fruit with well-defined or dif-fuse stripes were subjected to a chi-squared test with the significance set at $P < 0.05$, to verify the expected Mendelian segregation pattern of 3:1 in the F2 population. The linkage analyses were performed using the JoinMap (version 2.0) program (Stam and Van Ooijen, 1995), and the values obtained by the recombination frequencies were converted to maps of genetic dis-tance (in centimorgans) using the Kosambi function (Kosambi, 1944). A BLAST of the primer sequence and its PCR products was performed on the genome of this species, in relation to the stripe patterns on its fruit.

RESULTS

The five microsatellite markers (MCPI_04, MCPI_05, MCPI_11, MCPI_16, and MCPI_26) used to confirm hybridization were capable of discriminating the F1 population from its parents: individuals of the F1 population only exhibited the alleles of the feminine genitor (BRS Opara) and of the masculine genitor (Pérola).

During the phenotyping of the F1 and F2 populations and the parents for the external stripe patterns on the fruit, it was found that the parents were homozygous for this characteristic, because 100% of the examined fruits of cv. BRS Opara had well-defined stripe patterns and 100% of the fruits of cv. Pérola exhibited diffuse stripe patterns. In the F1 population, 100% of the fruits had well-defined stripes; in the F2 population, 150 (75%) of the fruits had well-defined stripes and 50 (25%) had diffuse stripe patterns. The observed values of the F2 population are equal to those that were expected for a monogenic and dominant character. The study of inheritance genetics carried out on the F2 population indicated that the presence of well-defined stripes is conditioned by a dominant gene, which segregates at a ratio of 3:1.

Only 12 of the 116 microsatellite markers examined were polymorphic in the parents of the population studied. During the analysis of ten individuals from the F2 population, only two primer pairs exhibited the expected allelic pattern, with the presence of an allele in the fruits with a well-defined stripe pattern and an absence of an allele in the fruits with a diffuse stripe pattern. The primer pairs were as follows: MCPI_05 (ATTTCTGGCCCCAGTGTAAG/GA ACAACGCAACCACGTATG) and MCPI_16 (TGCTCAATCCACCCTTTCTC/AAAAACA GCAACTCTCCCATC), developed by Joobeur et al. (2006). During the genotyping of the 200 individuals of the F2 population, the markers revealed the presence of an allele in the fruits with well-defined stripe patterns and the absence of an allele in the fruits with a diffuse stripe pattern (Figure 1).
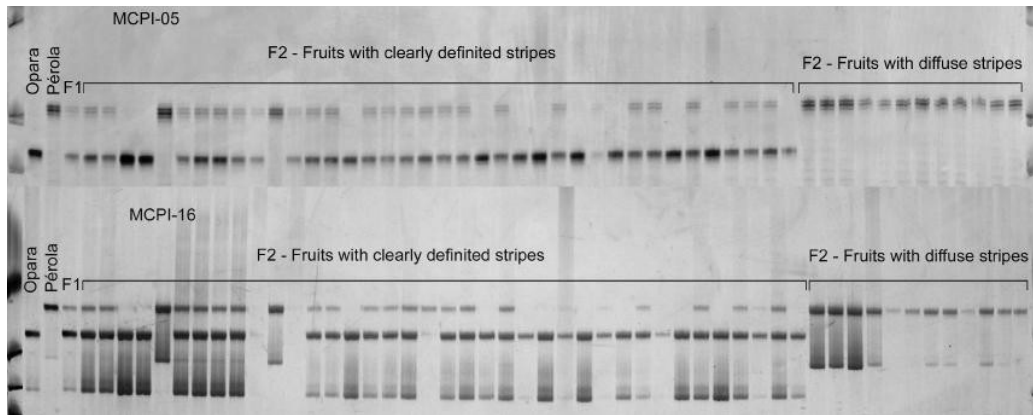

Figure 1. Allelic patterns of the watermelon cultivars BRS Opara (fruits with well-defined stripes) and Pérola (fruits with diffuse stripes) of F1 and of 50 individuals from the F2 population (38 with well-defined stripes and 12 with diffuse stripes), using two microsatellite markers (MCPI_05 and MCPI_16) that are associated with the stripe pattern of watermelon fruit.

The linkage analyses performed using JoinMap 2.0 revealed that the microsatellites MCPI_05 and MCPI_16 are linked to the gene responsible for the stripe pattern of watermelon fruit at a distance of 1.5 and 1.81 cM, respectively, with LOD scores of 39.28 and 38.11, respectively. The BLAST search of the two primer pair sequences and their PCR products performed on the watermelon genome, revealed that the gene related to the stripe patterns on watermelon fruit is located on chromosome six, and that the two markers in question are very close to each other, and to some genes predicted by Guo et al. (2013).

DISCUSSION

The results for obtaining the F1 and F2 populations indicate that the PMC method described by Dias et al. (2001) is effective in obtaining watermelon crosses in which the genitors have to be controlled and are free from pollen contamination by other genotypes. The microsatellite markers MCPI_04, MCPI_05, MCPI_11, MCPI_16, and MCPI_26 could be used as identification tools in parentage analysis of watermelon hybrids, in crosses among the cultivars 'BRS Opara' and 'Pérola'. Furthermore, such markers could also be used to distinguish 'BRS Opara'

from the hybrid obtained in this study, because both are mildew resistant and have a well-defined stripe pattern. In addition to exhibiting a slightly different green skin tonality, it is also important to point out that the hybrids cannot possibly be identified with absolute certainty by using only morphological descriptors, because skin coloration can be influenced by environmental factors.

Using molecular markers, the selection of the external pattern of the fruit can even be conducted before the fructification stage, at the plantlet stage. The frequent use of the marker in genetic studies, including paternity tests, is due to the fact that it is co-dominant, multiallelic, highly reproducible, and has a high resolution (Varshney et al., 2005; Oliveira et al., 2006).

The results obtained from the phenotyping of the F1 and F2 populations and the parents for the stripe pattern followed the Mendelian segregation of 3:1 (well-defined stripes:diffuse stripes), with a dominant gene that confers the well-defined stripe pattern. Therefore, it can be concluded that the heredity of this characteristic is monogenic and dominant. From the genotyping of the F2 population using the microsatellite markers MCPI_05 and MCPI_16, it was revealed that these are intimately linked to the locus that confers the characteristic stripe patterns on watermelon fruit. These markers could be used in MAS to select this characteristic in watermelons still in the plantlet stage. It is not necessary to wait until the fructification stage to select the pattern of the desired fruit, and there is the advantage of being able to identify homozygous and heterozygous individuals for this characteristic. Such considerations would save time, space, and money, because they allow the breeder to include only those plants that show the desired characteristic for an improvement program.

Few studies have used molecular markers in relation to phenotypic traits in the water-melon. Lin et al. (2009) identified a random amplified polymorphic DNA (RAPD) marker that is linked to resistance to *Fusarium oxysporum* f. sp *niveum*, and based on this, a sequenced characterized amplified region (SCAR) marker was developed, which, according to the au-thors, is able to quickly and accurately identify genotypes of watermelon that are resistant and susceptible to *Fusarium*. Wechter et al. (2008) studied the genetic expression of the wa-termelon fruit during maturation, in three stages: green pulp (start of development), pink pulp

(start of maturation), and red pulp (mature fruit) using microarrays, and identified genes that are possibly related to the maturation events of the fruit. Prothro et al. (2012) identified a quantitative trait locus (QTL), named M-QTL, in the linkage group (LG 2) as a candidate for MAS for watermelon seed size.

Studies involving molecular markers associated with phenotypic traits have also been developed in other members of the Cucurbitaceae, such as melon and cucumber. Some studies have associated molecular markers with phenotypic traits, both for resistance to disease and for various fruit traits. Liu et al. (2010) analyzed 700 combinations of sequence-related ampli-fied polymorphism (SRAP) primers, and identified a marker linked to the gene that confers resistance to *Podosphaera xanthii*, at a distance of 3.9 cM. Liu et al. (2010) cloned, sequenced, and converted this marker into SCAR. The authors stated that this marker can be used in MAS in the development of watermelon cultivars, in order to achieve resistance to *P. xanthii*. Meng et al. (2012) analyzed 130 plants of an F2 population from a cross of two cucumber geno-types, B-2-2 (long-fruit shape) and Y-3 (round-fruit shape), and identified SRAP markers that are linked to cucumber shape. Napier et al. (2009) identified a RAPD marker and an orange pulp-coloration marker in watermelon fruit that are linked to the QTLs for the high level of beta-carotene.

Studies that associate phenotypic traits with molecular markers in the watermelon are at an early stage. Following the characterization of the genome of this species by Guo et al. (2013), various other traits of interest could be associated with molecular markers, such as the soluble solid content, skin and pulp color, yield, acidity, precocity, and resistance to disease and pests, among others. The microsatellite markers MCPI_05 and MCPI_16 that were identified in this study as being linked to the stripe pattern in watermelon fruit can be used in MAS for the development of cultivars of this crop. Furthermore, the identification of the chromosome on which these markers are located will allow a closer study of the genes that are found in the proximity of these markers, which may be related to the stripe pattern of watermelon fruit.

ACKNOWLEDGMENTS

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