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Transferable polymorphic microsatellite markers from *Capsicum annuum* to *Capsicum baccatum*

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Abstract

Several researches have extensively used microsatellite markers for genetic mapping, because of their high transferring values among species from the same genus. This work aims at evaluating transferability of microsatellite locus developed to *Capsicum annuum* in a mapping population of *Capsicum baccatum*. We have tested 152 pairs of microsatellite primers developed to *C. annuum* in a mapping population constituted by 203 individuals F_2 originated from crossing between two accessions of *C. baccatum* var. *pendulum*, accomplishing a previous optimization of primers annealing temperatures. On the map construction, we have employed only the polymorphic markers and that generated clear marks, totalizing 62 markers transferred with success, 42 mapped, resulting in a percentage of 40.8% transferred microsatellites. Furthermore, we observed a relevant correspondence between microsatellite markers allocation on linkage groups *C. baccatum* and linkage groups *C. annuum.* This is the first report on the transfer of microsatellite markers to a species of *C. baccatum*, being an important advance on for the implementation of marker-assisted selection in breeding programs and also allows advances in more studied species such as *C. annuum.* Considering the slowness and higher cost of the processes of isolation and specific indicators development for microsatellite analysis, the results obtained at this work allows an improvement of the genetic mapping process of *C. baccatum* species.

Keywords: Molecular markers; SSR Primers; Transferability; Genetic map; Pepper.

Introduction

Capsicum genus includes chili and peppers, and it has great nutritional and economical relevance, and it is largely cultivated around the world (Haque and Gosh, 2018; Guzmán et al., 2020). Brazil is considered a center of diversity of this genus, presenting domesticated, semi-domesticated and wild species (Moscone et al., 2007). *Capsicum* genus has 42 species, including the new described ones like *C. caatingae*, *C. longidentatum* e *C. eshbaughii* (Barboza et al., 2019) Among these species, five are considered cultivated (*C. annuum*, *C. chinense*, *C. frutescens*, *C. baccatum*, e *C. pubescens*) (Moscone et al., 2007). *Capsicum* has a complex taxonomy and, generally, we identify its classification based on leaves, flowers and fruits characteristics (Olantunji and Afolayan 2018; Brilhante at al., 2021).

Capsicum is one of the main cultures in countries of tropical climate, however, despite this relevance, this is not a highly studied genus when we compare it with another cultivated plant from Solanaceae family, like tomato (*Solanum lycopersicum* L.), potato (*Solanum tuberosum* L.), and tobacco (*Nicotiana tabacum* L.) (Albrecht et al. 2012). Although, pepper is an important crop, the number of polymorphic molecular loci detected for the genus is far behind from many other cultivated plant species (Ince, Karaca, and Onus 2010).

Capsicum has species with 24 and 26 chromosomes. In accord with more specific terms, *C. baccatum*, object of this study, is diploid, with 24 chromosomes (2n=2x=24) (Moscone et al., 2007). The species *C. baccatum* has its center of diversity in Bolivia, where probably is also the center of its origin, what occurred about 4500 years ago (Moscone et al., 2007).

The species *C. baccatum* is divided into three botanical varieties: the domesticated *C. baccatum* var. pendulum, and semi-domesticated *C. baccatum* var. *baccatum* and *C. baccatum* var. *umbilicatum* (Carrizo García et al., 2016). In Brazil, *C. baccatum* var. *pendulum* is very appreciated and is represented by 'dedo-de-moça' (lady finger) peppers, with an elongated shape, and by 'cambuci' or 'chapéu-de-frade' (bishop's crown) pepper, with a shape like a bell and, due to the absence of pungency, is considered sweet pepper (Almeida et al., 2020). Despite its great genetic diversity, all studies target *C. annuum* (Rodrigues et al., 2012; Guzmán et al., 2020), due to the species higher commercial appeal.

Several researches on the study field of cultivated and wild species genetics have used molecular markers of microsatellite type (SSR - Simple Sequence Repeats), *e.g*., construction of genetic maps (Mimura et al. 2012; Wang et al. 2017), assessment of populational genetic parameters (Zhang et al., 2016; Azevedo et al., 2019), identification of parental origin, evaluation of improved population germplasm and assessment of similarity or genetic diversity (Villela et al. 2014; Jesus et al. 2019; Noor et al. 2020; Rabuma et al 2020).

Regarding to *Capsicum baccatum* var. *pendulum*, it is important to highlight that 183 marks comprise the species' genetic map, of which 42 are from microsatellite loci (Moulin et al. 2015a). Microsatellites are the most widespread marks for the QTL mapping on animal and vegetable species. Moulin et al. (2015b) describe initial QTLs for eleven characteristics of relevant agronomic interest necessaries to the improvement of *C. baccatum* var. *pendulum*, which include QTLs for production components.

The identification and development of microsatellite markers have allowed to increase significantly the density of linkage maps obtained using other markers, besides allowing phylogenetic studies and the development of different species (Thumilan et al., 2016). Microsatellite markers may function as anchors between parent's maps, allowing the construction of consensus maps or comparative mapping, and make possible greater robustness of the maps that share these markers in the same positions (Biswas et al. 2020).

An alternative to enable microsatellite analysis on species for which ones there are no specific primers yet is the primers transposition from evolutionarily related species (Laindorf et al., 2019). Several studies indicate similarity in DNA sequences located in regions at the borders of repetitive sequences, constituents of SSRs, between different species, indicating that these SSR primers can be transferred between related species. This greatly reduces the cost and time required for research (Carvalho et al., 2015). There are no microsatellite markers developed for the *C. baccatum* species and, so far, there are no other works describing the transfer of microsatellite markers to this species.

Accurate information on the transferability of microsatellite primers for *C. baccatum* would allow greater knowledge and use of this genetic resource. In this sense, this study aimed to test the transposition of 152 microsatellite markers developed for the *C. annuum* genome in *C. baccatum*. The transferability of the SSR markers studied in this work would be extremely useful for pepper breeding and mapping studies.

Results and discussion

We have observed polymorphism in the population of *C. baccatum* var. *pendulum* using primers designed to *C. annuum* (Figure 1). Regarding the microsatellite markers, from the 152 microsatellite markers available to *C. annuum* and tested in *C. baccatum*, 62 were successfully transferred and considered polymorphic, and 42 were mapped, resulting in a percentage of 40.8% microsatellite markers transferred.

We could not find a good transferability of some microsatellite primers, because there was no good sharpness in the obtained DNA bands. In other cases, there was an amplification and, therefore, the initiator transference, but we did not detected polymorphism. Although part of microsatellite primers was not transferred (59.2%), it is evident that the regions flanking the microsatellite remain conserved between species. Furthermore, this study is the first to validate these markers in the *C. baccatum* species, which enables synteny studies within the *Capsicum* genus.

There was a good correspondence between the allocation of microsatellite markers in linkage groups *Capsicum baccatum* var. *pendulum*, obtained in our work and demonstrated at the linkage map developed by Moulin et al. (2015a) and the *C. annuum* linkage group obtained by Minamiyama et al. (2006). A percentage of 66.67% of microsatellite markers mapped in our study were common to the linkage groups constructed by Minamiyama et al. (2006). This high similarity has a great relevance for synteny studies and comparative mapping with other species of *Capsicum*. According to Amorim et al. (2008), microsatellite markers enable the classification of genotypes in an efficient manner, in accord with their genealogy, origin and classification. In addition, microsatellite regions are conserved, there are not easily influenced by environmental conditions (Juliandari et al., 2019). Among the 62 transferred SSR markers only 20 were not mapped, which may be associated with the absence of segregating markers in some genomic regions, avoiding strong links between markers with distant positions or a marker distortion (Zhang et al. 2012).

The characterization of polymorphic microsatellite markers used in our study, including repeating type, pair sequence primers, optimal annealing temperature, obtained linkage group, and size of the expected product (bp), as well as a comparison with the linkage map for *C. annuum* constructed by Minamiyama et al. (2006) is available on Supplementary Table 1.

Part of the microsatellite markers were not transferred (41.8%), which according to Ajinath et al. (2021) may be associated with single or few nucleotide polymorphisms at the primer annealing site. The absence of an amplification product for any given genotype-marker combination indicates the presence of genotypes with null alleles in the given locus (Guzmán et al., 2020).

In accordance with the obtained data, Carvalho et al. (2015) conducted a study on genetic diversity of *C. frutescens* L. and *C. chinense* Jacq., through the microsatellite characterization developed for *C. annuum*. They found out that 19 (16.3%) microsatellite primers were transferred and polymorphic in *C. frutescens* L. and 36 (31.0%) in *C. chinense*.

It should also be noted that *C. frutescens* and *C. chinense* belong to the same gene complex as *C. annuum*, the *C. annuum* Complex. Classification performed according to the genetic relationship and crossability (reproductive capacity) between species of the complex. While *C. baccatum*, belongs to the *C. baccatum Complex* (Scaldaferro, 2019; Almeida et al., 2020), presenting a greater genetic distance and capacity of crossing much inferior to the species that are included in the same complex. In this work, it was observed a high transferability rate of microsatellites to *C. baccatum*, which was 40.8%, being an important synteny information for the construction of genetic maps in species of the genus *Capsicum*. These data reinforce the potential impact of this work, mainly for further studies between different gene complexes, and can also contribute in a valuable way to studies of genetic diversity and phylogeny.

Ince et al. (2010) obtained transferring rates higher than our work and similarly higher than Carvalho et al. (2015)'s study.

Table 1. Comparison between the percentage of microsatellite markers transferred to some species and the rate for *C. baccatum* obtained in our work.

Species	SSR Species	Transference (%)	Author Map
Cucumis melo	*Citrullus lanatus*	23.0	Ritschel et al. (2004)
Triticum aestivum	*Secale cereale*	25.0	Kuleung et al. (2004)
Hordeum vulgare	*Oryza rufipogon, O. sativa*	42.1	Varshney et al. (2005)
Capsicum spp.	*C. annuum*	75.5	Ince et al. (2010)
Brassica carinata	*B. rapa*, *B. oleracea*, *B. nigra* e *B. napus*	4.1	Priyamedha et al. (2012)
Solanum elaeagnifolium	*S. lycopersicun, S. tuberosum, S. melongena*	37.14	Zhu et al. (2012)
Capsicum frutescens and *C. chinense*	*C. annuum*	16.3 31.0	Carvalho et al. (2015)
Cucurbita spp. *Cucumis melo* *Citrullus lanatus*	*Cucumis sativus*	*6.9* 45.0 18.5	Natenuch et al. (2020)
Capsicum spp.	*Momordica charantia*	8.3	Ajinath et al. 2021
Capsicum baccatum	*C. annuum*	40.8	This work

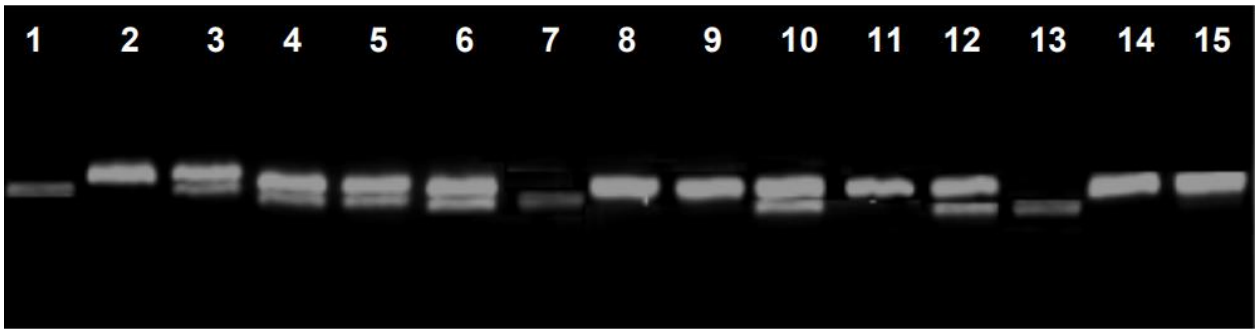

Figure 1. Metaphor agarose gel (4%). The amplification of DNA fragments using the CAMS-263 SSR marker. (1) P_1, (2) P_2, (3) F_1, e (4-15) F_2.

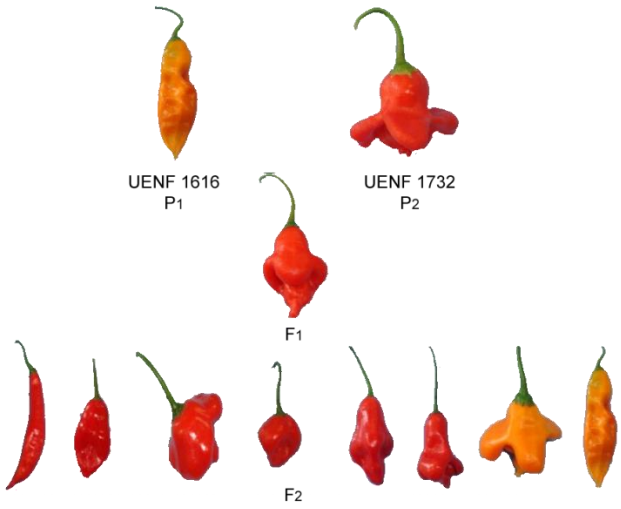

Figure 2. Fruits of parents, F_1 progeny and sample of the variability in F_2 generation originated from the cross between UENF 1616 (P_1) x UENF 1732 (P_2) used to constitute the genetic mapping population of *C. baccatum* var. *pendulum*.

In Ince's work, 75.5% of microsatellite primers were polymorphic. However, the authors worked with interspecific and extremely different materials of *Capsicum*, and not with a mapping population. Zhu et al. (2012) conducted a study to determine the transferability of 35 microsatellite primers available for nightshades as potatoes, tomatoes and eggplant in *Solanum elaeagnifolium*, species that also does not have available microsatellite primers. Among these, 13 primer pairs (37.14%) were polymorphic in *S. elaeagnifolium.*

The results obtained in our research are more favorable than those obtained in Priyamedha et al. (2012)'s study, which tested the transferability of 359 microsatellite primers designed to *Brassica rapa*, *B. oleracea*, *B. nigra* and *B. napus* for the construction of the first *Brassica carinata* species genetic map. Only 15 microsatellite primers were

successfully transferred, *i.e.*, the transferring rate was 4.1%, about ten times less than the transferring rate obtained in our study. Kuleung et al. (2004) also observed low transfer rates, studying the transferability of microsatellite primers synthesized for wheat in rye and vice versa and finding a wheat to rye transferability of 17%, while 25% of the markers designed for rye were polymorphic in wheat.

Ritschel et al. (2004) evaluated 67 primers developed for melon and tested in watermelon, both species of Cucurbitaceae family. They obtained 23% of transferability. Natenuch et al. (2020) evaluated 515 cucumber-derived SSR markers in other cucurbits. The transferability rate was 6.94% for pumpkin, 45.05% for melon and 18.55% for watermelon. Varshney et al. (2005) tested the transferability of 185 microsatellite markers developed for barley in rice and obtained a percentage like our research. They found out the successful transference of 78 microsatellite markers, making it possible to detect polymorphism in this culture. Ajinath et al. (2021) when studying the transferabilty of microsatellite markers developed for *Capsicum* in plants of the genus Momordica, they also observed a low transfer rate between species, only of 8.3%. As in this study, these authors also used microsatellite markers developed by Minamiyama et al. (2006) for the species *C. annuum*. In Table 1, it is possible to see descriptions of these different works and their respective percentage of transferred microsatellite markers, as well as the results of our study.

We mapped microsatellite markers in practically all linkage groups, except the group 10 (Moulin et al., 2015a) (Supplementary Table 1). Mimura et al. (2012) constructed a genetic map composed by 253 markers (151 SSRs, 90 AFLPs, 10 CAPSs and 2 TAGs), which has mapped microsatellite marks in all linkage groups. Barchi et al. (2007) developed a map for *C. annuum* in which were integrated 40 microsatellite brands concentrated in a few linkage groups, demonstrating that these markers were not well distributed throughout the genome.

It was possible to observe in our study that the distribution of markers was not uniform in the linkage groups; however, they were present in almost all linkage groups. The largest number of microsatellite markers were mapped on linkage group 4, corresponding to six marks, four of these mapped to the same linkage group of Minamiyama et al.'s work. (2006). To *C. annuum*, that has high density maps already described, it is possible building maps that are just saturated with microsatellites (Mimura et al., 2012; Sugita et al., 2013). However, for species such as *C. baccatum*, obtaining genetic maps is hampered by the availability and transferability of molecular markers, especially microsatellites. Information about transferability and use of microsatellites for *C. baccatum* species still very scarce in the literature. Studies on the transferability and construction of linkage maps, incorporating these markers of codominant nature, represent a valuable strategy for genomic studies on species.

Material and methods

Plant materials

From the crossing of accessions of *Capsicum* UENF 1616 (P1) x UENF 1732 (P_2), belonging to the Germplasm Bank of the State University of North Fluminense Darcy Ribeiro (UENF), the generations P_1 (female parent), P_2 (parent) were evaluated. male), F_1 (first hybrid generation) and F_2 (F_1 self-fertilization first generation) (Figure 1) (Bento, 2012).

Thus, the mapping population consisted of 203 F2 individuals. The accessions UENF 1616 (P_1) and UENF 1732 (P_2) come from the municipality of Campos dos Goytacazes, Rio de Janeiro, Brazil. The first has pungent, orange-colored fruits in the intermediate stage and red-colored when ripe. The parent UENF 1732 is characterized as very pungent, with a red colored fruit when ripe, with little accentuated aroma (Bento et al., 2009). Plants from these generations (P_1, P_2, F_1 and F_2) (Figure 2) were cultivated under field conditions in the Research Support Unit (UAP) of the Center for Agricultural Science and Technology (CCTA) of UENF and leaf samples from all the individuals were collected, identified and stored in an ultra-freezer to carry out this experiment.

Extraction and quantification of DNA

We have transferred about 300 mg of macerated leaf tissue to 1.5mL tubes and immersed in liquid N_2 for DNA extraction, according to Doyle & Doyle (1987) protocol, with modifications as described below. Firstly, we have added to the 1mL tubes some samples from preheated extraction buffer, containing 2% CTAB, 1.4 mol L^{-1} NaCl, 20 mmol L^{-1} EDTA, 100 mmol L^{-1} Tris-HCl (pH 8.0), 1% PVP and 0.2%-mercaptoethanol. Secondly, we have added 5μL of proteinase K (10 mg mL^{-1}) in each sample. We have incubated this material at 37°C for 30 minutes, gently mixing each 10 minutes. Posteriorly, we incubated it again at 65°C for 30 more minutes.

Then, we have centrifuged the samples at 8000g for 10 minutes. The supernatant (about 800μl) was transferred to a new properly identified tube and added to an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) to perform the deproteinization. We did some gentle inversions of this material during about 10 minutes until cloudy. Then, we separated the organic phase by centrifugation at 8000g for 10 minutes.

We transferred the supernatant to a new tube. Then, we added 200μL of NaCl at 2.0mol L^{-1} containing 4% PEG to a complete removal of protein and DNA recovery. We incubated the samples for 15 minutes at 4°C. We centrifuged the material at 8000g for 10 minutes, precipitated nucleic acids by adding two-thirds (400μl) of cold isopropanol volume and incubated it during 20 minutes at -70°C. Finally, we pelleted the precipitate by centrifugation at 8000g for 10 minutes. We discarded the supernatant and washed the precipitate twice with 200μl of 75% ethanol with ammonium acetate to salt removal (between each wash, the material was centrifuged at 8000g for 5 minutes). After discarding the last supernatant, we dried the material under natural conditions until remove the ethanol. Then, we re-suspended the material in 100μl of TE solution (Tris-EDTA – 10 mmol L^{-1} Tris-HCl, 1 mmol L^{-1} EDTA, pH 8.0) with RNase at a final concentration of 10μg mL-1 and incubated in a water bath at 37°C for 30 minutes. Afterwards, we stored the material at -20°C. We determined the quantification of DNA in a spectrophotometer, evaluating the quality by reasons A260 / A230nm and A260 / A280nm and agarose gel 1%.

Microsatellite markers

To study the transferability, we have selected 152 pairs of microsatellite primers (Supplementary Table 1) based on information available in the literature for mapping *C.*

annuum (Minamiyama et al., 2006; Barchi et al., 2007) and databases (http://solgenomics.net). We initially did a triage of the primer pairs with five genotypes (P_1, P_2, F_1, and two genotypes of F_2) to ascertain the generated polymorphism. Then, we used the microsatellite markers considered polymorphic and which generated sharp marks in the mapping population, constituted by 203 F_2 individuals.

We have conducted amplification reactions in a final volume of 21µL, containing the following reagents: 5 ng of genomic DNA, 0.75 unit of Taq DNA polymerase, 10X buffer (500 mM KCl, 100mM Tris-HCl, pH 8.3 Invitrogen), MgCl2 2.4 mM, dNTP (0.1 mM each deoxyribonucleotide) and 0.5µM of each primer. We have applied also 2µL of DNA and, subsequently, added 11µL of the abovementioned mixture. For staining, we added 8µL of red gel and blue juice mixtures in a concentration of 1:1.

On conducting of these amplification reactions (with Applied Biosystems thermocycler, Veriti model) we proceeded as follows. 1 min at 94°C for initial denaturation, followed by 35 cycles, each consisted of 94°C for 1 min, 55-63°C for 1 min (depending on the used primer), 72°C for 3 min and a final extension at 72°C for 7 min. We separated the amplified fragments using Metaphor high-resolution agarose gel 4% and subjected them to ultraviolet light for viewing (Minibis Pro – Bio-Imaging System manufacturer). We have performed a gradient test to determine the best annealing temperature in the initial triage. Then, we captured gel images for further analysis.

For the construction of the linkage map, we have used JoinMap software, version 4.0 (Van Ooijen, 2006), in which it was possible to establish the formed and ordered linkage groups, using a minimum LOD score of 3.0 and a maximum of 40% of recombination. Subsequently, we made a comparison between the microsatellite marks presented in the linking groups observed in this study with those obtained in a study proposed by Minamiyama et al. (2006).

Conclusions

The 62 transferred microsatellite markers prove the effectiveness of the transferability methodology of SSR loci between evolutionarily related species. Analyzing the high costs and the long time required for the development of specific SSR microsatellite analysis, the obtained results allow making a less costly and more efficient process of genetic mapping on *C. baccatum* species.

The transferability of polymorphic microsatellite markers is a major breakthrough for the implementation of assisted marker selection in *C. baccatum* breeding programs, and also allows the culture to benefit from advances in genetics and genomics of other most studied species, such as *C. annuum*. Part of microsatellite markers developed for the *C. annuum* genome could be successfully used for genetic analysis in *C. baccatum*.

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