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Cross-species Amplification of Microsatellite Loci Developed for *Passiflora edulis* Sims. in Related *Passiflora* Species

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ABSTRACT

*The aim of this study was to evaluate the selected 41 SSR markers developed for yellow passion fruit (*Passiflora edulis *f.* flavicarpa *Sims.) for their transferability to 11 different Passiflora species. Twenty-one SSR were successfully amplified in 10 wild species of passion fruit producing 101 bands. All the markers were amplifiable for at least one species. The mean transferability was 68,8%, ranging from 15,4% (primer PE11) to 100 % (PE13, PE18, PE37, PE41 and PE88). Transferability was higher for the species from the* Passiflora *subgenus than for those from the* Decaloba *and* Dysosmia *subgenus. The results indicated a high level of nucleotide sequence conservation of the primer regions in the species evaluated, and consequently, they could potentially be used for the establishment of molecular strategies for use in passion fruit breeding and genetics.*

Key words: Transferability, SSR, passion fruit

INTRODUCTION

According to Vanderplank (1996), the passion fruit belongs to the Passifloraceae family, which comprises 18 genera and approximately 630 species distributed in the tropical regions of America, Asia and Africa. The genus *Passiflora*, whose centre of origin is South America and centre of geographic distribution is the northern and central regions of Brazil, has the largest number of species in the family at approximately 400 (Vitta and Bernacci 2004). However, only approximately 60 species produce fruits with commercial value (Schultz 1968; Manica 1997), either for consumption or medicinal use. Approximately 90% of the species originated in the Americas (Lopes 1991) and more than 120 of them are native to Brazil (Cervi 1997; Souza and Lorenzi 2005).

Due to its economic and social importance, many studies have been undertaken to develop the varieties that are adapted to different cropping systems and climate conditions. Hence, activities related to the collection, conservation, characterisation and usage of *Passiflora* germplasm have been implemented to ensure that genetic variability is exploited in breeding programs. Due to its variability, wild species of *Passiflora* can contribute to the improvement of cultivated passion fruit in many different ways. In Brazil, a number of interspecific hybrids have been developed with the aims of (i) improving the

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agronomic traits, such as resistance to pathogens and cold tolerance; (ii) improving the physical, chemical and taste characteristics of the fruits for the fresh market, or the pulp for sweets, or ice cream; (iii) introducing characteristics, such as self-compatibility, because commercial species are self-incompatible; (iv) reducing the distance between the male and female flower structures to improve insect pollination; (v) contributing to day-length insensitivity; and (vi) developing ornamental hybrids (FAO 2009).

However, genetic variability can only be used efficiently if properly assessed and quantified. Thus, the germplasm must be characterised according to several morphological traits (Crochemore et al. 2003; Plotze et al. 2005; Bellon et al. 2009; Viana et al. 2010), agronomic behaviour (Cerqueira-Silva et al. 2008; Abreu et al. 2009) and molecular diversity (Cerqueira-Silva et al. 2009; Cerqueira-Silva et al. 2010; Viana et al. 2010), enabling advances in the description of genetic divergence among accessions.

Molecular markers have been used in plant breeding and in activities related to the conservation of genetic resources. SSR markers have been developed in *P. pohlii* (Pádua 2004), yellow passion fruit – *P. edulis* f. *flavicarpa* (Oliveira 2006), sweet passion fruit – *P. alata* (Pádua et al. 2005), *P. ovalis* (Kriedt 2009) and *P. cincinnata* (Cerqueira-Silva et al. 2012). This type of marker is highly polymorphic, co-dominant in nature, easy to score by the PCR and abundant in most organisms studied (Oliveira et al. 2006). However, the development of microsatellite markers is time consuming and a high cost activity (Gimenes et al. 2007). This limits the widespread use of microsatellite markers in this genus. A good alternative would be the use of a set of primers to obtain cross-species transferability.

In the transferability, or cross-amplification procedure, PCR primers developed for a studied (source) species are used to amplify microsatellites from closely (Isagi and Suhandono 1997; Harr et al. 1998; Cipriani et al. 1999) or sometimes quite distantly related species (White and Powell 1997; Roa et al. 2000; Zucchi et al. 2002; González-Martínez et al. 2004).

The objective of this study was, therefore, to test the ability of 41 passion fruit SSR primer pairs to amplify the PCR products from 10 species of the genus *Passiflora*. The transferable markers will immediately increase the pool of available SSR markers in wild relatives of passion fruit. These new markers will be useful in genotyping, characterising species relationships, the introgression of desirable alleles from wild germplasm pools and the application of marker-assisted breeding in passion fruit.

MATERIALS AND METHODS

Plant materials

Leaf samples from 11 *Passiflora* species including *P. edulis*, which is used as a check to confirm the size of SSR-fragments (Table 1) were harvested from the germplasm bank of Embrapa Cassava and Fruits, located at Cruz das Almas, Bahia, Brazil (12°39'59"S; 39°06'00"W). For each species, between three and five different germplasm plants were sampled.

Table 1 - Species evaluated using *P. edulis* microsatellite primers.

Species	Subgenus	Accession name	Number of plants
P. caerulea L.	*Passiflora*	BGM016	4
P. cincinnata Mast.	*Passiflora*	BGM322	5
P. edulis	*Passiflora*	BGM325	1
P. foetida L.	*Dysosmia*	BGM153	4
P. gibertii N. E. Br.	*Passiflora*	BGM008	5
P. gibertii N. E. Br.	*Passiflora*	BGM198	5
P. ligularis Juss.	*Passiflora*	BGM160	5
P. ligularis Juss.	*Passiflora*	BGM248	5
P. maliformis L.	*Passiflora*	BGM032	4
P. mucronata Lam.	*Passiflora*	BGM114	3
P. rubra L.	*Decaloba*	BGM117	5
P. setacea DC.	*Passiflora*	BGM241	5
P. setacea DC.	*Passiflora*	Pérola do Cerrado	5
P. suberosa L.	*Decaloba*	BGM152	5

DNA extraction, PCR amplification and SSR scoring

DNA isolation was conducted using the fresh leaves according to Doyle and Doyle (1990). A set of 41 SSR loci (Oliveira 2006) were evaluated and selected according to the quality of the amplification products. This resulted in 21 SSRs that were used in this work (Table 2). Polymerase chain reaction (PCR) was conducted in a 20 µl final volume, containing 10 ng of DNA template, 0.4 µM of each primer (reverse and forward) and 1.0 U Taq DNA polymerase (Invitrogen Co., Carlsbad, CA, USA). Annealing temperature, dNTP concentration, $MgCl_2$ and buffer conditions were optimised as shown in Table 3. The PCR products obtained using DNA from wild species were electrophoresed on 4% agarose 1000 (Invitrogen Co., Carlsbad, CA, USA) gels stained with ethidium bromide, or on 6% denaturing polyacrylamide gels stained with silver nitrate. The size of the fragments was estimated based on a 50 bp ladder (Biolabs).

SSR analysis

Polymorphic loci were characterised with regard to the number of alleles per locus (A), as well as expected (H_E) and observed (H_O) heterozygosities for each locus, and averaged over all loci using the Powermarker software (Liu and Muse 2005). A dendrogram for determining the relationship among the accessions was constructed using the software MEGA 4.0 (Tamura et al. 2007) based on the unweighted pair group mean average (UPGMA) method (Sneath and Sokal 1973) and the shared allele metric.

RESULTS

Transferability of SSR markers

Of the 41 passion fruit-specific SSRs assessed, 21 (51%) amplified characteristic, reproducible and high-quality bands in at least one *Passiflora* species. The other 20 markers (PE01, PE02, PE04, PE06, PE08, PE10, PE12, PE14, PE16, PE17, PE20, PE21, PE24, PE26, PE28, PE29, PE35, PE42, PE54 and PE60) amplified unspecific products and they were not used in this work. Table 2 shows the sequences of these primers along with the SSR repeat motif and annealing temperature.

Table 2 - SSR markers used in this study: locus code, sequence of primers, repeated motif, class, and size observed in passionfruit *Passiflora edulis* Sims f. *flavicarpa* Deg (Oliveira 2006).

Locus name	Forward sequence (5' – 3')	Reverse sequence (5' – 3')	Repeated motif	Class	Size (pb)
PE03	gcagcgagggaagaaaaa	tgagacatcgtgcgtgaa	$(GA)_{10}$	Interrupted	156
PE07	tgctcattgatggtgcttg	tcgtctcttctcctccttca	$(GA)_{23}$	Interrupted	138
PE09	ggaaatccgaaaactggttg	gggcctttatccatgtttga	$(AT)5(AC)_8$	Compound	268
PE11	gcataagttgtcggtcttgg	cctcgaacctctatcatcca	$(GT)_{11}$	Perfect	178
PE13	aagcaccccaatcgttga	ccccctgccacctgagta	$(GT)_6$	Interrupted	172
PE15	accgttaaatccaagcaagt	aaatgcaaaagaatgatatgtta	$(CTTTAGC)_5$	Imperfect	204
PE18	ccgtgaaccaaccatttctc	ttgcagcacaaacaagtcaa	$(TG)_9$	Perfect	220
PE19	ttaacaggacttagcacttga	ctcatccttcttccatctttg	$(CA)_{14}$	Perfect	245
PE23	caatcccttgacccataga	cgtccatccttctccttt	$(GA)_{19}$	Perfect	206
PE27	ttgctcattgcactcatcct	gcagacatttcctggagca	$(GT)_7$	Perfect	139
PE37	caaaaggataggcctgatgtc	tgcttggtcatccactgaag	$(TG)_8$	Perfect	232
PE38	gatcggtcctcggttagac	agtcacacagcatgagaaatc	$(TG)_8$	Perfect	215
PE41	atcggggttcgcttatttg	cgttcatcctttagtgggcta	$(TTAA)_5$	Interrupted	220
PE58	gcaatttcaccatcttctgct	ccacggtcatggatgttc	$(AC)_{11}$	Perfect	243
PE59	gaacacttcgcatggctaga	ttccgaatcaaaccgtaact	$(ATCTA)_3$	Perfect	276
PE64	atcaattacgcaccccaaac	ggaacgtcaatcaagtgagga	$(AC)_8$	Perfect	228
PE66	ccatagtcccaacaagcatc	gctgtggaccctaactcagtc	$(AC)_9$	Perfect	165
PE74	ccctcttatcaatagcgttgg	gcacgagcacgagtatttatt	$(ATCACA)_5$	Interrupted	215
PE75	cacaatcggtgggaaagata	gtagttttgggcagtttgc	$(TG)_{17}$	Perfect	178
PE88	cttcagggtcacacacatt	gttcatcctttagtgggct	$(TTAA)_6$	Interrupted	293
PE90	tcaggaagattgcatgttagt	ctgggttttgtttatgttgc	$(AGC)_5$	Perfect	245

Table 3 - Annealing temperature (TA°C), dNTP, buffer and $MgCl_2$ concentration used for each primer, as well as characteristics of SSR primers developed for *P. edulis* and cross-amplified in 10 species (14 accessions) of *Passiflora* species.

Locus	TA (°C)	dNTP (mM)	Buffer	$MgCl_2$ (mM)	Number of alleles	Allele range	Gene diversity	PIC
PE03	60	0,2	1X	1,5	2	140-150	0.50	0.37
PE07	60	0,2	2X	1,5	11	90 -140	0.75	0.85
PE09	56	0,2	2X	1,5	10	205-280	0.75	0.71
PE11	60	0,2	2X	1,5	4	270-320	0.69	0.64
PE13	60	0,2	2X	1,5	2	165-175	0.15	0.15
PE15	60	0,35	2X	1,5	9	160-220	0.58	0.77
PE18	60	0,35	2X	1,5	13	170-240	0.70	0.86
PE19	52	0,2	1X	2,5	7	225-270	0.75	0.72
PE23	56	0,35	2X	1,5	5	130-200	0.77	0.73
PE27	60	0,2	1X	2,5	2	145-150	0.18	0.16
PE37	60	0,2	1X	1,5	1	240	0.00	0.00
PE38	56	0,2	1X	2,5	5	220-280	0.74	0.68
PE41	60	0,2	1X	2,5	2	220-225	0.43	0.28
PE58	60	0,2	1X	1,5	3	300-365	0.41	0.37
PE59	56	0,35	2X	1,5	6	250-280	0.69	0.66
PE64	56	0,2	2X	1,5	2	150-240	0.39	0.31
PE66	60	0,2	1X	2,5	5	240-270	0.77	0.67
PE74	62	0,2	1X	2	5	180-260	0.75	0.69
PE75	60	0,35	2X	1,5	3	140-160	0.58	0.34
PE88	60	0,35	2X	1,5	2	285-290	0.00	0.32
PE90	60	0,2	1X	2,5	7	210-280	0.73	0.77

Seven primer pairs (PE07, PE13, PE18, PE19, PE37, PE41 and PE88) amplified the PCR products for all of the *Passiflora* species but not in all plants of the same species. Only primers PE37 and PE41 produced PCR amplicons in all plants. Eleven primers showed more than 70% transferability in *Passiflora* accessions. Three markers PE11, PE38 and PE58 showed transferability < 25%. Primers PE11, PE23, PE38, PE59, PE64 and PE90 only amplified sequences in the species that belonged to the subgenus *Passiflora* but did not amplify the sequences in the subgenera *Decaloba*, or *Dysosmia*; thus, these primers presented an interesting marker for taxonomic distinction. Considering only species from the *Passiflora* subgenus, successful cross-amplification was higher than 73%. However, when considering only species from the *Decaloba* subgenus, the transferability ratio was approximately 54%.

The accession BGM032 (*P. maliformis* – subgenus *Passiflora*) exhibited the highest observed transferability (100%), while BGM117 (*P. rubra* – subgenus *Decaloba*) exhibited the smallest transferability (42.9%). A remarkable diversity was observed within the accessions because the amplification ratio varied among the plants of the same accession and among accessions. For example, the observed transferabilities of the *P. ligularis* accessions ranged from 0 (plants of BGM160) to 100% (plants of BGM248) for the primer PE03. The transferability ratio for each accession is presented in Table 4.

SSR variability from the *Passiflora* species

Considering the polymorphic primers, the PIC values ranged from 0.15 (PE13) to 0.86 (PE18), with a mean value of 0.60 (Table 3). The number of alleles detected per locus varied from 1 to 13 (mean: 6.2), while the number of genotypes per locus ranged from 1 to 16 (mean: 6.1).

The primer pair PE59 amplified products exclusively for the species belonging to the *Passiflora* subgenus, but the plants of the *P. ligularis* and *P. setacea* subgenera produced no PCR product. Similarly, considering the primer PE23, amplification was observed only for the *Passiflora* subgenus species, with the exception of *P. ligularis* and *P. gibertii*.

A dendrogram was constructed using the shared allele distance and the UPGMA method (Fig. 1). The species were split into two groups, one comprising the species from the *Passiflora* subgenus and the other from the species that belonged to the *Decaloba* and *Dysosmia* subgenera.

Table 4 - Transferability observed for a set of 21 primers pairs developed to *P. edulis* in 10 species (14 accessions) of *Passiflora* species.

Specie	Accession	Primers – code PE																				
		03	07	09	11	13	15	18	19	23	27	37	38	41	58	59	64	66	74	75	88	90
P. ligularis Juss.	BGM160	-	+	+	-	+	+	+	+	-	-	+	-	+	-	-	-	+	-	+	+	+
P. ligularis Juss.	BGM248	+	+	+	-	+	+	+	+	-	-	+	-	+	+	-	+	+	-	+	+	+
P. foetida L.	BGM153	-	+	+	-	+	+	+	+	-	+	+	-	+	-	-	-	+	+	+	+	-
P. rubra L.	BGM117	-	+	+	-	+	-	+	+	-	-	+	-	+	-	-	-	+	-	-	+	-
P. setacea DC.	BGM241	-	+	+	-	+	+	+	+	+	+	+	-	+	-	-	+	+	+	-	+	+
P. setacea DC.	*Pérola do Cerrado*	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	-	+	+
P. suberosa L.	BGM152	+	+	+	-	+	-	+	+	-	-	+	-	+	+	-	-	-	+	+	+	-
P. cincinnata Mast.	BGM322	-	+	+	-	+	+	+	+	+	+	+	-	+	-	+	-	+	-	+	+	+
P. maliformis L.	BGM032	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
P. caerulea L.	BGM016	-	+	+	-	+	+	+	+	+	+	+	-	+	-	+	+	+	-	+	+	+
P. gibertii N. E. Br.	BGM008	-	+	+	-	+	+	+	-	-	-	+	-	+	-	+	+	+	-	-	+	+
P. gibertii N. E. Br.	BGM198	+	-	+	-	+	+	+	+	-	-	+	-	+	-	+	+	+	+	+	+	+
P. mucronata Lam.	BGM114	-	+	-	-	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+

\+ denotes high quality amplification; - denotes low quality or no amplification

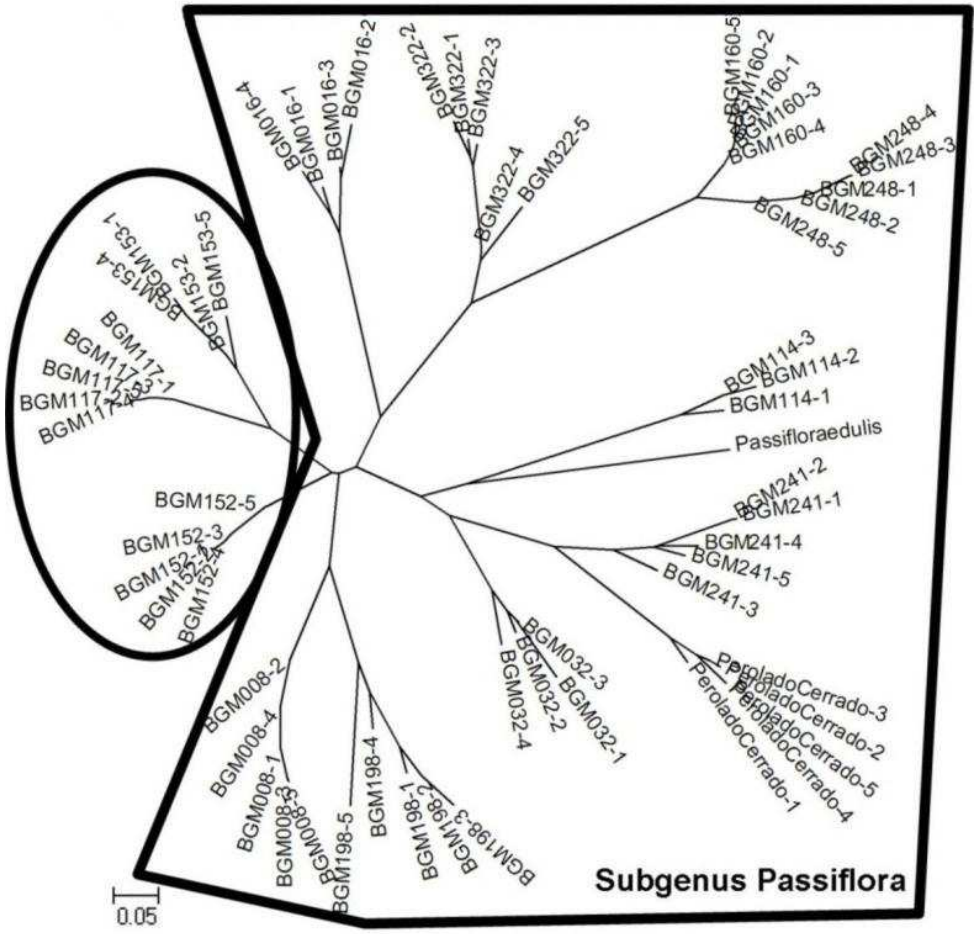

Figure 1 - Clustering of *Passiflora* genotypes derived from a unweighted pair group mean average (UPGMA) cluster analysis using the Shared allele coefficient based on passionfruit SSR primers.

In the *Passiflora* subgenus group, the accessions were clustered according to the species to which they belonged because there were subgroups comprising exclusively by the accessions of *P. gibertii*, *P. cincinnata*, *P. ligularis*, *P. setacea*, *P. mucronata* and *P. caerulea*. For *P. gibertii*, *P. setacea* and *P. ligularis*, in which more than one accession was analysed, all the plants of the same accession grouped together but constituted different subgroups related to the accession to which they belonged. In the non-*Passiflora*-subgenus group, the accessions were distributed into the homogeneous groups according to their species.

For all the accessions of the species that did not belong to the *Passiflora* subgenus, a considerable number of primers (PE59, PE23, PE64, PE90, PE38, PE11 and PE03) did not amplify any product.

DISCUSSION

This study provided an important first step in the development of a set of microsatellite markers suitable for multispecies studies of *Passiflora*. The primers were tested across a reasonable range of conditions for each species (e.g., varying $MgCl_2$ and dNTP concentrations and annealing temperatures), and results, therefore, provided the information required for more extensive investigations of polymorphisms. The high cross-species transferability and levels of polymorphisms of microsatellite loci indicated that the application of these markers was a worthwhile and cost-effective approach. Although transferability was in general very high, the number of polymorphic SSRs that amplified the high-quality products was lower, at approximately 53%. This result was in agreement with Barbará et al. (2007), who reviewed 64 primer, representing 611 cross-species. These authors found that in the plants, the transferability of polymorphic markers exhibited a success rate close to 60% in eudicots.

Generally, the transferability of SSRs isolated from the cultivated species to the wild species decreases as the phylogenetic relationship of the wild species decreases. The results obtained for the genera *Manihot* (Roa et al. 2000), *Arachis* (Koppolu et al. 2010), *Allium* (Lee et al. 2011) and *Citrus* (Luro et al. 2008) were in agreement with the previously established phylogenetic relationships. In this study, even when considering the species belonging to different phylogenetic clades, good transferability was obtained because all the species could be amplified by a minimum of 49% of the primer pairs tested.

The transferability ratio was higher in the species that belonged to the subgenus *Passiflora* than for that of the *Decaloba*, or *Dysosmia* subgenera. While the transferability ratio for the subgenus *Passiflora* was 0.73, for species that did not belong to this subgenus, the index was 0.54. Because the source of the markers used in this study was *P. edulis*, a species that belonged to the subgenus *Passiflora*, this result was clearly explained by the genetic relationship among the species employed.

It was possible to detect the variability within the accessions that was related to allelic size, or to non-amplification. Similarly, the set of transferable markers was able to distinguish the accessions because all the plants for the same accession were clustered together.

The species were split into two major groups. The large group was composed of the species that belonged to the subgenus *Passiflora*, while the small group comprised the species from the subgenera *Decaloba* (*P. suberosa* and *P. rubra*) and *Dysosmia* (*P. foetida*). However, the genetic distance between *P. rubra* and *P. foetida* was lower than that between *P. rubra* and *P. suberosa*, the two species of the *Decaloba* subgenus.

In addition, possibly due to a high mutation rate in the SSRs (Oliveira et al. 2006), these markers were capable of distinguishing the organisms at the species level but not at higher hierarchical taxonomic levels, such as the subgenus.

However, because the aim of this study was to detect the markers developed for *P. edulis* that could be used to reveal diversity, assist in the breeding programs, mainly for hybrid detection, construct genetic maps based on interspecific crosses, exploit the diversity of the primary gene pool, or assist in recurrent selection proceedings, the inability of these markers to distinguish among the subgenera did not constitute a problem.

The possibility of transferring this set of SSR markers that exhibited very high-quality products would provide an efficient and cost-effective platform for the establishment of molecular strategies for use in the passion fruit breeding and genetics.

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