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ISSR and SSR markers for determining genetic relationships among three wild species of *Passiflora*

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ABSTRACT. *Passiflora cristalina*, *Passiflora miniata* and *Passiflora coccinea* are wild species with similar floral characteristics, especially color and floral structure, as well as the color of their fruits. Due to their similarities, mainly the floral characteristics, these species are often confused in the field. Given that hybridization is commonplace between *Passiflora* species in the same region, hybrids could result from crosses involving mainly *P. coccinea*. We examined genetic distance and possible hybrid nature across *P. cristalina*, *P. miniata*, *and P. coccinea*, via ISSR and SSR markers. Genomic DNA was extracted from leaf samples of five *Passiflora* species (*P. cristalina, P. coccinea, P. miniata, P. setacea*, and *P. edulis*), the latter two being used as witness species. Following quantification, the amplification conditions were tested and optimized. Eighteen ISSR primers presented satisfactory amplification products, with 81 bands being amplified and 99% polymorphism. Through genetic distance and cluster analysis, *P. cristalina* and *P. coccinea* were found to be genetically close, while *P. miniata* remained in an isolated cluster, nevertheless with low dissimilarity with *P. cristalina*. Twenty-three SSR primers were tested, of which 18 were polymorphic. There was a high transferability rate, 95.65%, demonstrating that genetic proximity between tax is directly related to successful transferability. The main coordinates, genetic distance and

cluster analyses showed a clear separation of species presenting similar floral characteristics (*P. cristalina*, *P. coccinea*, and *P. miniata*) from the remaining ones used as controls (*P. edulis* and *P. setacea*). SSR markers identified *P. cristalina* as a possible natural hybrid between *P. miniata* and *P. coccinea*.

Key words: Wild species; Molecular markers; Genetic resources

INTRODUCTION

The Passifloraceae family is predominantly found in American and African tropical and subtropical regions (Cervi, 2006); it includes18 genera; the *Passiflora* genus is the most species rich, comprising from 521 to 537 species (Feuillet and Macdougal, 2004; Vanderplank, 2007). Four genera occur in Brazil, as follows: *Ancistrothyrsus* Harms, *Dilkea* Mast., *Mitostemma* Mast., and *Passiflora* L., totaling 150 species, of which 87 are endemic (Flora do Brasil, 2020, under construction).

New *Passiflora* species are still being described; about 90% of these species are native to the Americas (MacDougal, 2011). *Passiflora miniata*, described by Vanderplank (2006), belongs to the subgenus *Passiflora*, *Coccinea* supersection, and originates and is distributed in the Amazon region (Peru, Brazil and Colombia) and in the Guianas (Lim, 2012). It presents a red-color flower with three series of purple corona filaments, with small fruits that have mottled green and cream colors (Vanderplank, 2006).

Passiflora cristalina belongs to the *Diasthephana* supersection of the subgenus *Passiflora* and is found in Cristalino State Park in northeastern Mato Grosso state. It presents red flowers with two series of red-to-pink corona filaments, and small fruits that have mottled dark green and cream colors (Vanderplank, 2011).

Passiflora coccinea is native to the Guianas, Venezuela, the Amazon region of Peru, Bolivia and Brazil; it belongs to the subgenus *Passiflora*, *Diasthephana* supersection, presenting scarlet-red flowers with two series of violet or pinkish-white corona filaments, and fruit peel of mottled green color with longitudinal stripes (Vanderplank, 2000).

The three species present great morphological similarity, especially in color and flower structure, as well as similar fruits, with the number and color of the series of corona filaments as their major differences (Vanderplank, 2006). They are wild species and may be considered as repositories of genes of interest, though they have been little studied.

Wild species have attracted the attention of breeders due to their genetic potential, since they have disease and pest resistance genes, besides agronomic traits of interest (Junqueira et al., 2005; Meletti et al., 2011); however, hybridization with cultivated species is not always feasible (Hajjarand Hodgkin, 2007). For successful hybridization, parental species need to be genetically closely related (Pereira et al., 2005). There are several techniques available to study genetic similarity among species, among which are molecular markers, which are highly variable and allow estimation of genetic distances between species at the DNA level (Faleiro et al., 2008).

Among molecular markers, ISSR (Inter Simple Sequence Repeat) markers are considered as informative in genetic diversity studies (Bornet and Blanchard, 2001). A number of authors reported high polymorphism levels in *Passiflora* species when using such markers (Santos et al., 2011; Costa et al., 2012; Sousa et al., 2015).

As for microsatellites, or SSR (Simple Sequence Repeats), these have emerged as the most widely used molecular markers due to their high information content (Oliveira et al., 2006). In spite of being species-specific, the potential transferability of primers across species of the same genus enables their use (Bravo, 2006).

The objectives of our study were: i) to estimate genetic distances between five species of the *Passiflora* genus (*P. cristalina*, *P. miniata*, *P. coccinea*, *P. edulis*, and *P. setacea*) using ISSR and SSR markers; ii) to assess the potential transferability of microsatellite markers for detecting polymorphisms in *Passiflora* genotypes; iii) to ascertain *P. cristalina* hybrid nature by comparing band patterns by means of SSR molecular markers.

MATERIAL AND METHODS

Plant material

Leaf samples of five *Passiflora* species (*P. cristalina*, *P. coccinea*, *P. miniata*, *P. setacea*, and *P. edulis*), were used, with the latter two used as witnesses.

DNA extraction

Genomic DNA extraction was carried out using the Qiagen*DNeasy Plant* Mini Kit, following methodology described by the manufacturer. For the analysis using ISSR markers, DNA extraction was carried out in bulk using 10 individuals per species. For the analysis using SSR markers, extraction was carried out individually using 5 individuals per species, and upon confirmation of absence of allelic difference, 1 individual per species was then selected for analysis. Following extraction, DNA integrity and quantification were assessed in 1.0% agarose gel and then DNA was diluted to 5 ng.μL^{-1}.

Amplification conditions and statistical analysis for ISSR

Eighteen ISSR primers were used for the analyses. The volume of amplification reactions was 13 µL, with 6.08 µL ultrapure water, 1.3 µL PCR (1X) Buffer, 1.5 µL dNTPs, 1.0 µL magnesium chloride, 1.0 µL primer, 0.12 µL Taq polymerase enzyme, and 2 µL genomic DNA, using a 100 pb Lambda marker. Polymerase chain reactions (PCR) were carried out in a thermal cycler under the following conditions: 5 min at 94ºC (initial denaturation), followed by 35 cycles at 94ºC for 1 min, 46-52°C for 1 min, 72ºC for 3 min, and a final extension at 72ºC for 7 min.

Amplified fragments were separated in 2% agarose gel, stained with a RedTM gel and Blue Juice (1:1) mixture and exposed to UV light (Minibis Pro documentation system – Bio-imaging System) in order to view the results. Polymorphisms were tabulated based on presence (1) or absence (0) of bands.

Dissimilarity analysis was performed through a binary matrix by using Rogers and Tanimoto's Simple Matching Coefficient and the cophenetic correlation coefficient (CCC). Cluster analysis via dendrogram was performed by means of the UPGMA method with the aid of Mega software version 6 (Kumar et al., 2009).

Amplification conditions and statistical analysis for SSR

Twenty-three microsatellite primer pairs developed and optimized for *P. edulis* (Oliveira, 2006), *P. alata*; (Pádua et al., 2005), and *P. setacea* (Cerqueira-Silva et al., 2014) were tested. All PCR amplifications were performed in a thermal cycler, and the volume of amplification reactions was 13 µL, with 6.08 µL ultrapure water, 1.3 µL PCR (1X) Buffer, 1.5 µL dNTPs, 1.0 µL magnesium chloride, 1.0 µL primer, 0.12 µL Taq polymerase enzyme, and 2 µL genomic DNA, using a100 pb Lambda marker. PCRs were performed as follows: 4 min at 94ºC for initial denaturation, followed by 35 cycles, each consisting of 94ºC for 1 min, 52-64°C for 1 min, 72ºC for 3 min, and a final extension at 72ºC for 7 min.

Fragments were separated in 4% metaphor gel, stained with a Red^{TM} gel and Blue Juice (1:1) mixture and exposed to UV light (Minibis Pro documentation system – Bio-imaging Systems) in order to capture images.

A matrix of numerical data was built that was assigned values from 1 to the maximum number of alleles found per locus. Genetic distance was calculated with the aid of Genes software (Cruz, 2013), by using Smouse and Peakall index. Clustering analysis via dendrogram was performed by means of the UPGMA method with the aid of Mega software version 6 (Kumar et al., 2009).

The mean number of alleles per polymorphic locus (Na), the effective number of alleles (Ne), Shannon Index (I), expected (He) and observed (Ho) heterozygosity were calculated per locus using Genalex 6.3 software (Peakall e Smouse, 2012). The dispersion graph was based on Principal Coordinate Analysis (PCoA) using the Genalex 6.3 software (Peakalland Smouse, 2012).

Identification of *P. cristalina* hybrid nature through SSR markers

Since *P. cristalina* may be a natural hybrid of *P. coccinea* and *P. miniata*, hybrid identification was tested by visual analysis of band patterns derived from this possible parental combination. Molecular markers generated by the different primers were analyzed for the presence or absence of informative bands showing clear patterns of single band easily mapped to the possible parents. Informative bands are marks present in parent 1 and absent in parent 2, and presence of both in the supposedly hybrid genotype (*P. cristalina*) confirmed natural hybridization. Only highly clear and reproducible bands were considered as informative bands.

RESULTS AND DISCUSSION

ISSR Markers

Fifty-five ISSR primers were tested, 18 of which generated satisfactory amplification products in the study species. The number of amplified bands per primer ranged from 3 to 7, showing evident power to detect polymorphisms (Table 1). A total of 81 ISSR bands were amplified, of which 79 were polymorphic, with four bands per primer on average, which is an expected result when using interspecific analysis (Fajardo et al., 1998; Santos et al., 2011).

Table 1. ISSR primers used in a genetic diversity study of five *Passiflora* species in Campos dos Goytacazes.

Primer Identification	Sequence (5'-3')	Annealing Temperature	No. of amplified loci	No. of polymorphic loci
1	$(GA)_8CC$	48ºC	7	7
2	$(GT)_6CC$	48ºC	4	3
7	$(AC)_8CT$	48ºC	4	4
17	$(AC)_8T$	48ºC	5	5
19	$(AG)_8YA$	48ºC	5	5
20	$(GA)_8YT$	48ºC	5	5
23	$(CA)_8CYG$	48ºC	4	4
32	$(AG)_8C$	48ºC	5	4
33	$(AG)_8T$	48ºC	3	3
40	$(AC)_8CTT$	48ºC	3	3
50	$(AC)_8C$	48°C	5	5
51	$(ATC)_6$	48°C	5	5
57	$(GA)_9T$	48ºC	4	4
59	$(AC)_4Y$	48ºC	7	7
70	$(GA)_7RC$	48ºC	3	3
72	$(GTG)_4RC$	46ºC	5	5
73	$(CA)_7YC$	48ºC	4	4
7M	$(ATC)_6$	46ºC	3	3

There are few reports on the use of ISSR markers in *Passiflora*, however, some authors reported high polymorphism level in their work with species of this genus using such markers. Santos et al. (2011) evaluated 45 *Passiflora* accessions (three *P. alata* accessions and 42 *P. edulis* accessions) using 18 ISSR primers and obtained 227 polymorphic bands with 12.61 bands per primer on average. Costa et al. (2012) characterized 63 genotypes of sour passion fruit vine in Embrapa's Manioc and Fruit Production program and obtained 22 polymorphic primers generating 266 bands with 11.56 bands per primer on average. Sousa et al. (2015) evaluated 25 wild species of *Passiflora* from the UESC germplasm bank in Ilheus, Bahia, using ISSR markers and obtained 20 polymorphic primers among the 31 tested, with a total of 331 bands and 16 bands per primer on average.

Four main groups were identified: Group I comprised *P. cristalina* and *P. coccinea* species and Groups II, III and IV were constituted of *P. miniata*, *P. setacea*, and *P. edulis*, respectively (Figure 1). The cophenetic correlation coefficient (r) was 0.7693, which is a suitable coefficient, since values of $r \geq 0.56$ are considered ideal, thus reflecting consistency with the genetic distance matrix values (VazPatto et al., 2004).

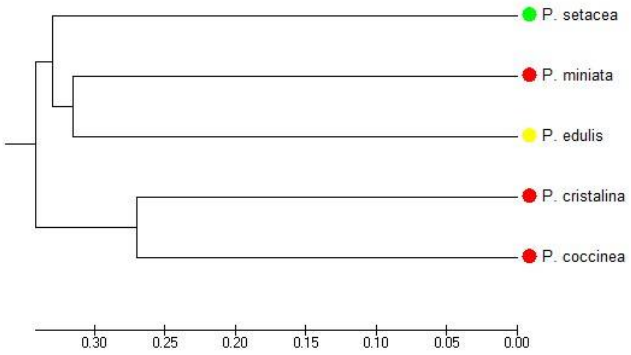

Figure 1. Dendrogram obtained by UPGMA clustering method complemented with Rogers and Tanimoto's Simple Matching Coefficients across five species of the *Passiflora* genus based on ISSR markers.

Passiflora cristalina and *P. coccinea* were identified as the least dissimilar, presenting a value of 0.54; this result was expected, since these two species are morphologically similar and belong to the same genus, sub-genus and supersection. As for *P. miniata*, which remained as an

isolated group, it presented a genetic distance coefficient of 0.59 from *P. cristalina* and of 0.67 from *P. coccinea*, therefore, closer to *P. cristalina*. On the other hand, *P. coccinea* and *P. setacea* were the ones that presented the highest similarity, with a coefficient equal to 0.74 (Figure 1).

SSR Markers

Among the 23 SSR primers tested, 18 were considered as polymorphic, which is equivalent to a transferability rate of 95.65% (Table 2). This was an expected result, since the study species belong to the same genus; i.e. they present the same primer sites flanking these conserved regions, demonstrating that the evolutionary proximity between taxa is directly related to successful transferability of SSR markers. Therefore, transferability analysis is highly convenient as it reduces time and costs required for the development of these markers (Carvalho et al., 2015).

High cross-amplification rates in *Passifloras*, from SSR primers previously developed for two species, *P. edulis* (Oliveira, 2006) and *P. alata* (Pádua et al., 2005), were observed by Cerqueira-Silva (2012b) when they examined several wild species of *Passiflora* in their studies, with results of 86% transferability.

With respect to genetic diversity among the species that we evaluated, a low number of alleles were found in all polymorphic loci. The number of alleles per locus ranged from 2 to 3, with mean of 2.44, obtaining a total of 44 alleles for the 18 loci evaluated (Table 2).

Table 2. Characterization of 18 loci by molecular analysis via SSR markers.

Locus	*Primers*	aT
Pad 2	F:CACATTTGCCGTCACTGG R:CGGCATACGATAAATCTCCTG	60
P34	F:GGCAGGATATGCTTTGGTT R:GCTGTCGGACACATGGAC	60
P40	F:GAATCAATGGAACACAAGCA R:CCAGCCCACTAGACCACCT	60
P76	F:ACTCTCACCTCAATCGACC AATTGTTACTCCGTTTCTCTGA	60
Ps16	F:GAGAAAGCGAGTCAGCGAGA R:GACTCCAATATCGGCACTTCA	58
Ps3	F:GTAGCGTCTCGGCAGGTC R:ACTCTAAGTCGGCCACTCTTG	60
P43	F:CTCAGTGAGGAATAAGCAATCA R:ATTTGGCATGCTGTTACGC	60
Ps21	F:CCCAATCGCTGAGAGGAGT R:CGGTAGGCTCATTCGTGTCA	58
Ps5	F:TCGGTCTTCGTATTCAACTCTG R:GAGGAACTGGCATCGCAT	58
P96	F:GAATCAATGGAACACAAGCA R:CCAGCCCACTAGACCACCT	56
Ps2	F:TAGCTTAACACAATGCAACAGA R:CAACGGAGAACGATGTCAG	54
Ps1	F:TAGCTTAACACAATGCAACAGA R:CAACGGAGAACGATGTCAG	50
Ps7	F:ACAGGGGTGAGGCACATTC R:TCTGTTATTATCATCGGCAGG	56
Ps6	F:GTTGGATCAAAGGGTCACA R:CAACTACTGGATCGAACTGGTA	58
P90	F:TCAGGAAGATTGCATGTTAGT R:CTGGGTTTTGTTTATGTTGC	58
P25	F:GTGTTTGTGGCGATGTGATTA R:GACAAACGTTGTTTCCGCT	60
P74	F:CCCTCTTATCAATAGCGTTGG R:GCACGAGCACGAGTATTTATT	62
Ps4	F:CAACAGGAGGTGAGGTGTGA R:GACAGTGCAACTTTAGGCGAC	64

aT - Annealing Temperature

By means of the Principal Coordinate Analysis (PCoA), we observed that two coordinates accounted for 84.6% of total variation, which may be seen in the dispersion chart (Figure 2). A distance between *P. edulis* and *P. setacea* and the remaining species is noticed, which was expected, since these two species present distinct morphological characteristics. Nevertheless, proximity was observed between *P. edulis* and *P. setacea*, which is supported by a molecular phylogeny study in which conserved plastid sequences were used (Muschner et al., 2003), and by the Santos et al. (2014) study, which showed that they present good crossbreeding combining ability in both directions.

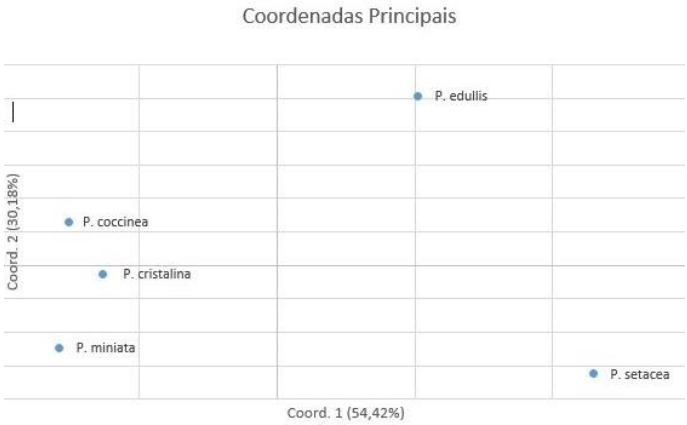

Figure 2. Genetic Distance between five species of *Passiflora* genus.

Formation of three groups could be observed, where three species presenting similar morphological characteristics (*P. cristalina*, *P. coccinea*, and *P. miniata*) were allocated to group I, and groups II and III comprised *P. setacea* and *P. edulis*, respectively (Figure 3).

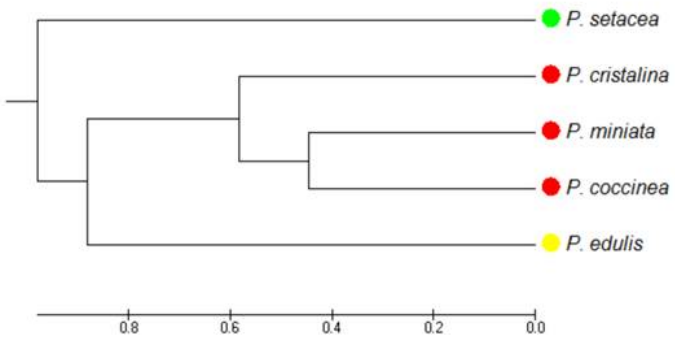

Figure 3. Dendrogram obtained by cluster analysis across five *Passiflora* species using the UPGMA clustering method.

The genetic distance ranged from 0.89 to 2.11, and this variation demonstrates the extensive diversity between these species. A smaller distance was observed between *P. coccinea* and *P. miniata* (0.89); this was an expected result, since they present similar traits

such as red flowers, and they are often erroneously cultivated due to their similarity (Vanderplank, 2006). Greater dissimilarity was observed between *P. setacea* and *P. coccinea* species (2.11).

In studies with *Passiflora* using joint analyses of morphologic descriptors by means of Ward-MLM procedure and SSR markers, (Paiva et al., 2014a,b), *P. setacea* and *P. coccinea* were allocated to different groups, as observed in our study.

Although *P. setacea* and *P. edulis* species were not allocated to the same group, they presented low dissimilarity (1.11). Santos et al. (2014), aiming at obtaining hybrids resistant to fruit hardening disease, performed interspecific hybridization between these species and found that such hybridization was successful in both crossbreeding directions, evidencing that there is genetic compatibility.

Two pairs of SRR primers were sufficient to identify the *P. cristalina* hybrid nature. Through analysis of SSR markers, *P. cristalina* was identified as a possible natural hybrid between *P. miniata* and *P. coccinea* due to the presence of two bands from each of the possible parents (Figure 4).

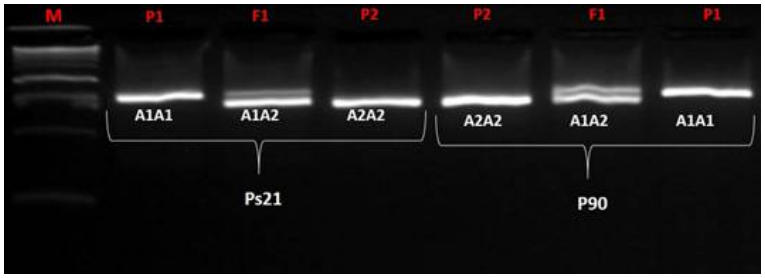

Figure 4. Electrophoresis analysis of DNA amplification products related to three wild species of *Passiflora*. P1 – parent 1 (*P. coccinea*); P2 – parent 2 (*P. miniata*); F1 – hybrid (*P. cristalina*); M – molecular marker; Ps1 and P90 – SSR primers.

This result is consistent with data obtained by cluster analysis using both dominant and co-dominant markers. By the cluster analysis based on ISSR markers, it was found that *P. miniata* did not cluster with *P. cristalina* and *P. coccinea*; however, it presented a low genetic dissimilarity with *P. cristalina* (0.59). In the cluster analysis using SSR markers, the three species remained in the same group, with a shorter distance between *P. coccinea* and *P. miniata* being observed.

The number and color of corona filaments are considered as a characteristic morphological marker among these species: *P. coccinea* has white filaments, while *P. miniata* has red filaments, and *P. cristalina* has pink filaments (Vanderplank, 2006; Zappi, 2011), and this may be considered an intermediate trait between theparents, suggesting interspecific hybridization.

Several methodologies may be used to confirm hybrids, from those based on morphological traits (Oliveira et al., 2005), to those done at molecular and cytogenetic levels. Molecular markers are excellent tools to confirm hybridization, by which the use of one or two primers or primer combinations with at least one informative band is sufficient for confirmation (Faleiro et al., 2003). Hybridization confirmation in *Passiflora* has been performed based on molecular markers that consist of a more reliable methodology to

confirm parenthood in hybrid passion fruit plants, such as RAPD (Junqueira et al., 2008; Conceição et al., 2011) and SSR (Santos et al., 2012) analyses.

CONCLUSIONS

Based on our results, we concluded that a) there is similarity among *P. cristalina*, *P. coccinea*, and *P. miniata*, estimated using both ISSR and SSR molecular markers; b) we found an increased transferability rate for SSR markers, evidencing that the evolutionary proximity between taxa is directly related to successful transferability; c) SSR markers were shown to be effective in identifying the hybrid nature of *P. cristalina*, which was considered, in this study, as a possible natural hybrid between *P. miniata* and *P. coccinea*.

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