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Cytogenetic characterization of the *Passiflora edulis* Sims × *Passiflora cincinnata* Mast. interspecific hybrid and its parents

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Abstract The genus *Passiflora* L. consists of approximately 530 widely distributed species, including *Passiflora edulis*, which has drawn interest because of its commercial and agronomic value. *Passiflora cincinnata* is another important species owing to its long flowering period and resistance or tolerance to diseases and pests. In the present study, the meiotic segregation and pollen viability of an interspecific hybrid (*P. edulis* × *P. cincinnata*) and its parents were analyzed. The genomic contents were characterized using chromomycin A_3 (CMA_3)/4′-6′-diamidino-2-phenylindole (DAPI) staining, fluorescent in situ hybridization with 5S/45S ribosomal DNA (rDNA), genomic in situ hybridization (GISH), and inter-simple sequence repeat (ISSR) markers. The results indicated the diploid chromosome number for the parents and interspecific hybrid was $2n = 18$. We also observed regular meiosis, one pair of 5S rDNA sites, and two pairs of 45S rDNA sites that co-localized with two pairs of CMA_3^+/$DAPI^-$ bands. The GISH data revealed three distinct chromosomal groups in the hybrid. The genetic origins of the interspecific hybrid, and its relationship with its parents were also confirmed using ISSR markers.

Keywords CMA_3/DAPI staining · In situ hybridization · ISSR · Meiotic behavior · Passion fruit

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Introduction

The genus *Passiflora* L. (Passifloraceae) consists of approximately 530 species (Ulmer and MacDougal 2004; Hansen et al. 2006), with approximately 140 distributed in Brazil (Cervi 2006). Many of these species are economically important, with some valued for their ornamental (Vanderplank 1996) and pharmacological properties (Tommonaro et al. 2007) in the cosmetics and food industries. The fruits from several species, such as passion fruit (*Passiflora edulis* Sims), can be consumed or processed to produce juices, ice cream, alcoholic beverages, and sweets. Brazil is one of the primary international producers of passion fruit (IBGE 2013), highlighting *P. edulis* as the species grown in more than 95 % of Brazilian orchards. However, this species is highly susceptible to various diseases, including bacteriosis, fusariosis, and viral

infections, which threaten the productivity of cultures. Consequently, breeders have attempted to genetically improve passion fruits through hybridizations (Meletti 2011; Cerqueira-Silva et al. 2014). These breeding efforts have involved the use of wild species to generate new cultivars that are genetically resistant to several diseases, which may influence productivity and fruit quality characteristics (Bellon et al. 2007).

Among the wild passion fruit species in Brazil, *P. cincinnata* Mast. may be an agronomically important species, especially because its fruits have a characteristic flavor desired by consumers (Araújo et al. 2008; Cerqueira-Silva et al. 2010). Moreover, this species exhibits variable agronomically relevant characteristics (Araújo et al. 2008), including disease tolerance (Siebra et al. 2016). This species is diploid ($2n = 18$ chromosomes), with two pairs of 45S ribosomal DNA (rDNA) sites. This is similar to *P. edulis* (Melo and Guerra 2003), which makes *P. cincinnata* potentially useful for hybridizations (Cerqueira-Silva et al. 2012).

Although there are reproductive barriers, several researchers have produced *Passiflora* hybrids using sexual and somatic hybridization techniques (Barbosa and Vieira 1997; Soares-Scott et al. 2003; Cuco et al. 2005; Barbosa et al. 2007; Junqueira et al. 2008). Interspecific hybrids have also been generated for ornamental purposes (Ulmer and MacDougal 2004; Conceição et al. 2011; Santos et al. 2012). However, to genetically improve species, clarifying the potential of the parents is crucial. Examining and characterizing interspecific hybrids through genetic and phenotypic means to estimate genetic diversity, viability, and fertility rates, as well as identifying pre and post-zygotic barriers are also important.

The chromosomal content and behavior of promising genotypes and hybrids can be examined using mitotic and meiotic analyses (Soares-Scott et al. 2003; Barbosa et al. 2007; Santos et al. 2012), as well as chromosome banding techniques (Moraes et al. 2013) and fluorescent in situ hybridization (FISH) (Souza et al. 2010; Moraes et al. 2013). Recently, genomic in situ hybridization (GISH), which involves genomic probes, helped identify chromosomes in interspecific hybrids and allopolyploid lines (Raina and Rani 2001) of *Epidendrum* L. (Moraes et al. 2013), *Spondias* L. (Almeida et al. 2007), and *Passiflora* (Melo et al. 2015) species.

Inter-simple sequence repeat (ISSR) markers may be useful for detecting molecular polymorphisms in improved and non-improved passion fruit germplasms (Costa et al. 2012) if they are able to generate highly reproducible results and produce an abundance of polymorphic fragments to identify intraspecific and interspecific variabilities (Dantas et al. 2012).

In the present study, the meiotic segregation and pollen viability of an interspecific hybrid (*P. edulis* × *P. cincinnata*) and its parents were analyzed. We also examined the respective genomic contents using chromomycin A_3 (CMA_3)/4′-6′-diamidino-2-phenylindole (DAPI) staining, FISH with 5S/45S rDNA, GISH, and ISSR markers. Our objective was to characterize genotypes potentially useful for passion fruit genetic improvement programs.

Materials and methods

Plant material and interspecific hybridization

Passiflora cincinnata (accession CPI54) and *P. edulis* (accession EBA55) individuals obtained from the Active Germplasm Bank of Passion Fruit of the Empresa Brasileira de Pesquisa Agropecuária (Embrapa Semiárido) were used in crosses to obtain the interspecific *P. edulis* × *P. cincinnata* hybrid. Floral buds from pre-anthesis female (*P. edulis*) and male (*P. cincinnata*) parents were covered with paper bags for 24 h before plants were used for controlled pollinations. After seven days, the permanency or abortion of the floral buds and initial fruit development were observed and recorded.

Parental seeds, which were generated by controlled pollinations, as well as seeds from six interspecific hybrid individuals, were sown in substrate containing vermiculite, washed sand, and agricultural soil (1:1:1). The germinated plantlets were then transplanted to plastic pots containing the same substrate, and incubated in a greenhouse at an air temperature of 27 ± 2 °C and a relative humidity of 70 %.

Cytogenetic analysis

Meiotic analysis and pollen viability

Young floral buds were collected, fixed directly in Carnoy solution (ethanol: acetic acid, 3:1) for 24 h at room temperature, and stored at −20 °C. To prepare slides, flower buds were washed in distilled water for 5 min, and then hydrolyzed in 5 N HCl for 5–10 min.

Anthers were macerated separately on slides containing 45 % acetic acid, and then frozen in liquid nitrogen, air dried, and stained with 2 % Giemsa. Cover slips were fixed into place with Entellan. Conventional chromosome staining with Giemsa proceeded as described by Guerra (1983). To assess pollen grain viability, samples were prepared on slides and stained with 2 % acetic carmine according to the procedure of Radford et al. (1974).

Mitotic analysis

Pre-treatment, fixation, and chromosome preparation

Root tips were collected, pre-treated with 2 mM 8-hydroxyquinoline for 24 h at 8 °C or 4.5 h at 20 °C, fixed in ethanol: glacial acetic acid (3:1) or methanol: acetic acid (3:1), and stored at −20 °C until used. Roots stored in fixative were washed two times in distilled water for 5 min each and hydrolyzed in HCl 5 N for 20 min for use in conventional analyses. For FISH and GISH chromosome banding analyses, root samples were digested with a 2 % cellulase solution (Onozuka Sigma) and 20 % pectinase (v/v) in a humidity chamber at 37 °C for 2.5 h.

Two methods were used to prepare slides depending on the fixative used. Roots fixed in ethanol or methanol: acetic acid (3:1) were macerated on a slide containing 45 % acetic acid, crushed between the slide and cover slip, and then submerged in liquid nitrogen. After removing the cover slip, slides were air dried and stained with 2 % Giemsa. Cover slips were then fixed in place using Entellan prior to conventional analysis. Alternatively, slides were stored at −20 °C. Slides were dried using an air pump, immersed in 45 % acetic acid for 12 s, and dried again at 37 °C. The best slides were selected through stained with a DAPI (2 µg/mL): glycerol (1:1, v/v) solution. Samples were then bleached in a fixative (ethanol: glacial acetic acid) for 30 min. Slides were immersed in absolute ethanol for 1 h at room temperature, air dried, and stored at −20 °C until used for CMA_3/DAPI staining, FISH, and/or GISH.

Staining with chromomycin A_3/4′-6′-diamidino-2-phenylindole

Staining with CMA_3 and DAPI fluorochromes was completed using the protocol described by Schweizer and Ambros (1994). Samples were stained with CMA_3 (0.5 mg/mL) for 1 h and DAPI (2 µg/mL) for 30 min, mounted in a McIlvaine-glycerol buffer, and stored in a darkened chamber for 3 days. Samples were then analyzed using a fluorescence microscope.

Obtaining probes

To localize the 45S rDNA sites, we used the SK18S and SK25S probes, containing *Arabidopsis thaliana* L. 18S and 25S rDNA (Unfried et al. 1989; Unfried and Gruendler 1990). The 5S rDNA was obtained using a polymerase chain reaction (PCR) based on the *P. edulis* genomic DNA sequence. We used the primers 5′-GTGCGATCATACCAGC(AG)(CT)TAATGCAC CGG-3′ and 5′-GAGGTGCAACACGAGGACTTCC-CAGGAGG-3′. The 5S and 45S probes were conjugated with digoxigenin-11-dUTP (Roche) and biotin 11-dUTP using a Nick Translation kit (Invitrogen) and a BioNick Labeling system (Invitrogen), respectively.

For GISH, genomic DNA of one of the parents was used as a probe, while DNA from the second parent was used as blocking DNA. Genomic DNA was extracted from fresh leaves to prepare the probe and blocking DNA according to a procedure described by Doyle and Doyle (1990), with some modifications. Extracted genomic samples were quantified in a 0.8 % agarose gel (w/v), with lambda DNA as the molecular weight standard. The *P. edulis* and *P. cincinnata* probes were conjugated with digoxigenin-11-dUTP as described, and used separately to analyze the metaphases of the interspecific hybrid and *P. edulis* (*P. cincinnata* probe) plants. The blocking DNA was fragmented in a water bath at 100 °C for 20 min. After conjugating with digoxigenin-11-dUTP, probes were stored at −20 °C for subsequent use in hybridizations. Probes were diluted 1:20 in DNA blocker solution.

In situ hybridization

Slides stored at −20 °C were pre-treated with an absolute ethanol: glacial acetic acid (3:1, v/v) solution, followed by dehydration in a series of ethanol solutions (70–100 %) and incubation at 60 °C for 30 min. for subsequent use in FISH and GISH experiments. The denaturation of chromosomes and probes, post-hybridization bath treatments, and sample detection step were completed according to the Heslop-Harrison et al. (1991) methods, with modifications described by Pedrosa et al. (2002).

Hybridization mixtures consisted of 50 % formamide (v/v), 10 % dextran sulfate (w/v), 2 × SSC, and 2.5–5 ng/μL probe. Samples were denatured at 75 °C for 7 min and incubated in a humidity chamber at 37 °C for 18 h for hybridizations. The biotin-labeled probes were detected using mouse anti-biotin (Invitrogen) and goat anti-mouse tetramethylrhodamine isothiocyanate (TRITC) (Sigma) in 1 % (w/v) bovine serum albumin. The digoxigenin-labeled probes were detected using sheep anti-digoxigenin-fluorescein isothiocyanate (FITC) (Roche) and anti-sheep-

Fig. 2 Chromosome banding (**a**–**c**), fluorescent in situ hybridization (**d**–**f**) and genomic in situ hybridization (**g**–**n**) in mitotic metaphases of *P. cincinnata* (**a**, **d**), *P. edulis* (**b**, **e**), and *P. edulis* × *P. cincinnata* hybrid (**c**, **f**–**n**). Chromosomes 6 and 8 showed CMA_3^+ bands in all genotypes analyzed (**a**–**c**), co-localized with 45S rDNA sites (*red*) (**d**–**f**). The 5S rDNA sites (*green*) are on chromosome 5 (**d**–**f**). GISH in *P. edulis* × *P. cincinnata* hybrid using the total genomic DNA of *P. edulis* (**g**–**j**) showed eight completely labeled chromosomes (j_1), six partially labeled chromosomes (j_2), and four non-labeled chromosomes (j_3). GISH using *P. cincinnata* probe (**k**–**n**) revealed four completely labeled chromosomes (n_1), six partially labeled chromosomes (n_2), and eight non-labeled chromosomes (n_3). *Bar* 10 μm. (Color figure online)

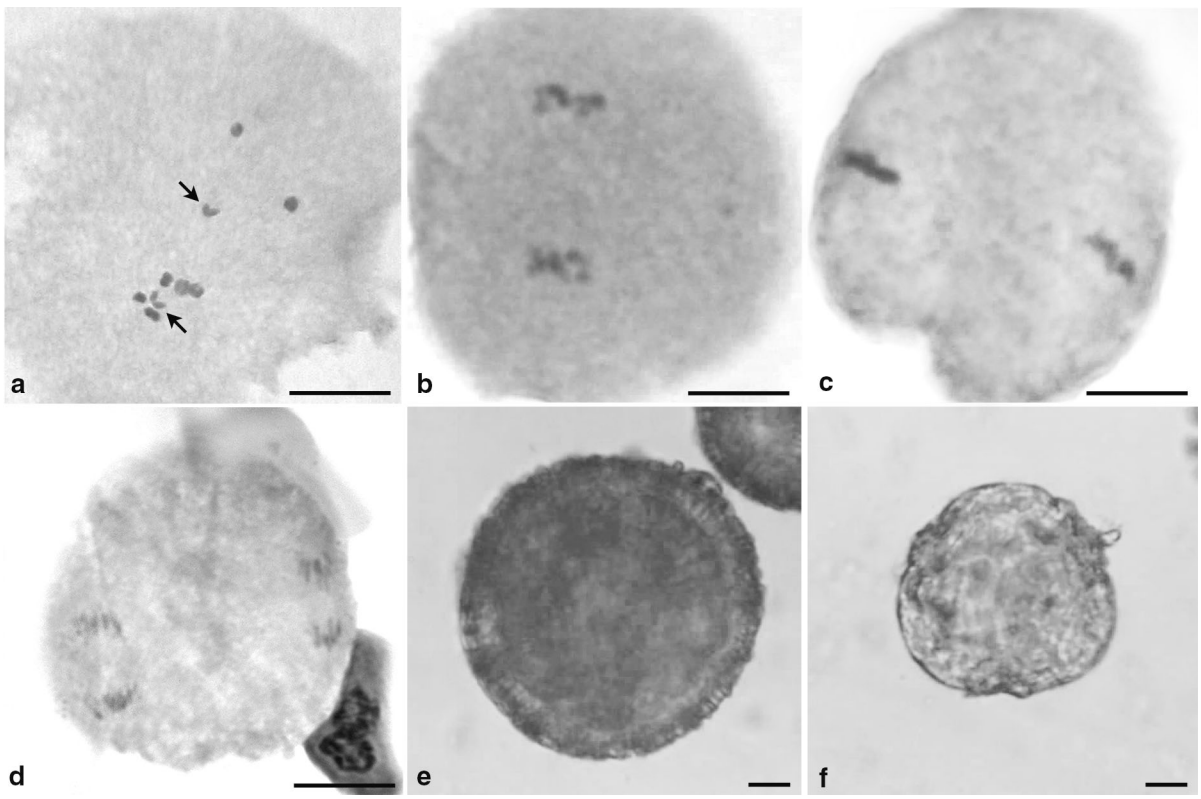
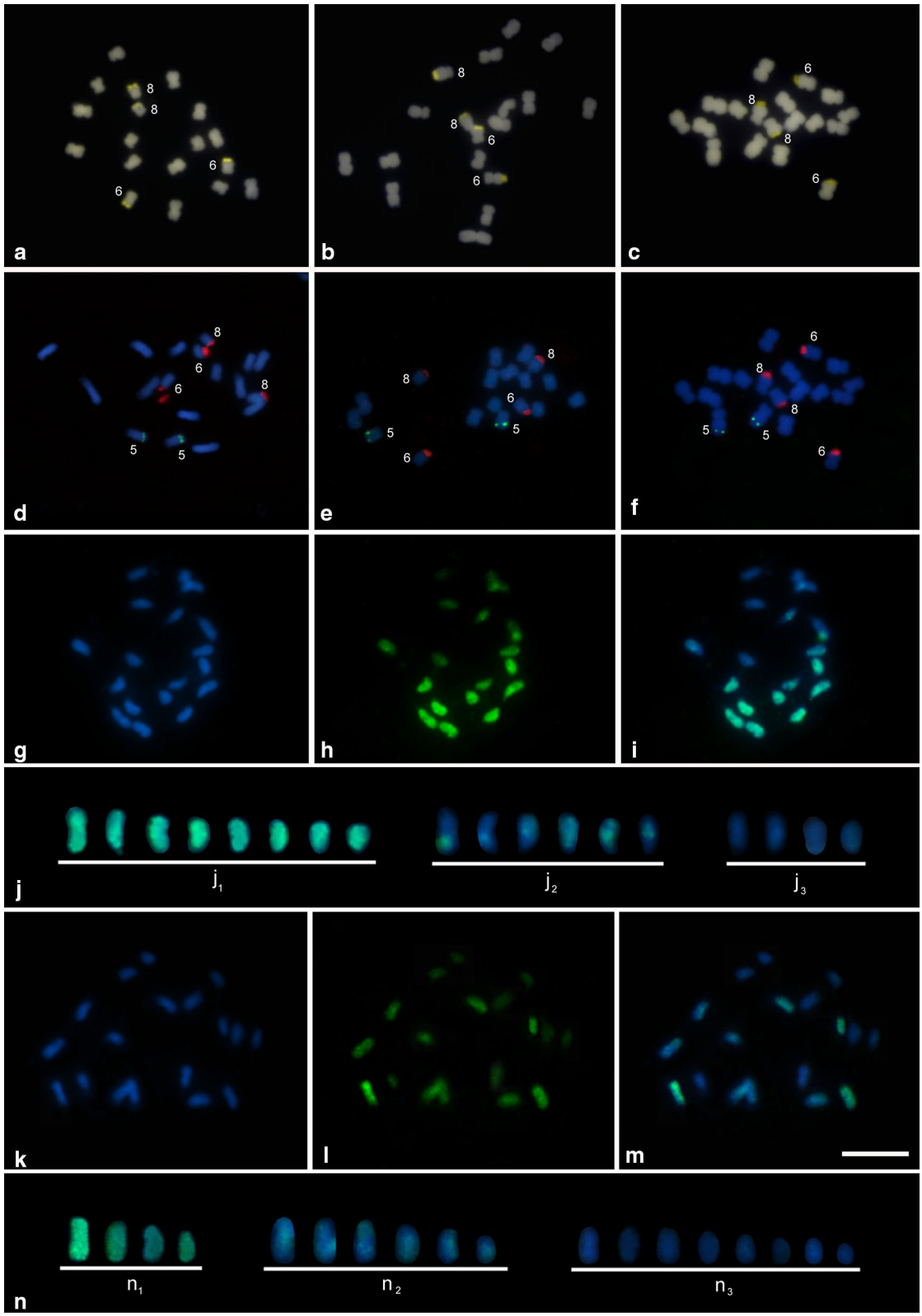


Fig. 1 Meiosis stability and pollen viability of the *P. edulis* × *P. cincinnata* hybrid: diplotene with nine bivalents, two of them (arrows) with partially paired chromosomes (**a**), regular anaphase I (**b**), metaphase II (**c**) and anaphase II (**d**); viable (**e**) and unviable (**f**) pollen grains. Bars are equivalent to 10 μm

Table 1 Examined species and interspecific hybrids of *Passiflora*, origin and average values of VP (pollen viability), VPGD (viable pollen grain diameter) and NVPGD (non-viable pollen grain diameter)

Species/hybrids	Origin	VP (%)	VPGD (μm)	NVPGD (μm)
P. cincinnata (CPI54)[a]	Genviniano—PI	96.9	82.64 ± 3.66	58.29 ± 2.94
P. edulis (EBA55)[a]	Cairu—BA	89.9	77.20 ± 2.95	57.26 ± 4.71
P. edulis × *P. cincinnata*	Interspecific crossing	89.8	76.93 ± 2.88	57.45 ± 4.09

[a] CPIB0554, EBAM0255 correspond to the codes in the BAG (Active Germplasm Bank)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fluorescein isothiocyanate (Sigma) in 1 % (w/v) bovine serum albumin. All samples were counterstained and mounted with 2 μg/mL DAPI and Vectashield (Vector).

Data analysis

Cells were analyzed using a Leica DM2000 epifluorescence microscope, and images of the best cells were captured with a Leica FX-350 camera using Leica QFish software. The images were optimized for contrast and brightness with Adobe Photoshop CS3 (Adobe Systems Incorporated). In meiotic analysis, ten slides per individual were analyzed, observing the meiotic behavior of chromosomes and the average percentage of meiotic irregularities in the stages of meiosis I and II. The minimum number of 50 cells was examined at each stage of meiotic divisions. The viable and non-viable pollen grains in five random fields from five slides were scored using a 20× magnification lens, for a total of about 800 grains. The average number of viable pollens was calculated, as well as their respective standard deviations. Additionally, the diameter of the viable and non-viable pollen grains was measured using the Dino Capture 2.0 program.

To identify chromosomes based on morphological characteristics, five metaphases were analyzed using the UTHSCSA Image Tool program (Donald et al. 2007). An ideogram was generated based on chromosome lengths using Adobe Flash CS6 Professional software. Based on chromosomal measurements, the following karyological parameters were estimated: length of haploid complement (LHC), absolute length of individual chromosomes (C), ration between arms (LA/SA), and karyotype formula. The chromosome nomenclature suggested by Guerra (1986) was used (e.g., metacentric and submetacentric). Chromosomes were arranged according to decreasing relative length (i.e., in relation to the length of the haploid complement) and identified.

Inter-simple sequence repeat markers

DNA extraction and quantification

We extracted DNA from fresh leaves according to a procedure described by Doyle and Doyle (1990), with some modifications. The DNA samples were quantified in a 0.8 % agarose gel (w/v) by comparing the intensity of sample bands with that of a lambda DNA control (Invitrogen). The DNA samples were diluted to 20 ng/μL in milli-Q H_2O and stored at −20 °C until used in a PCR assay.

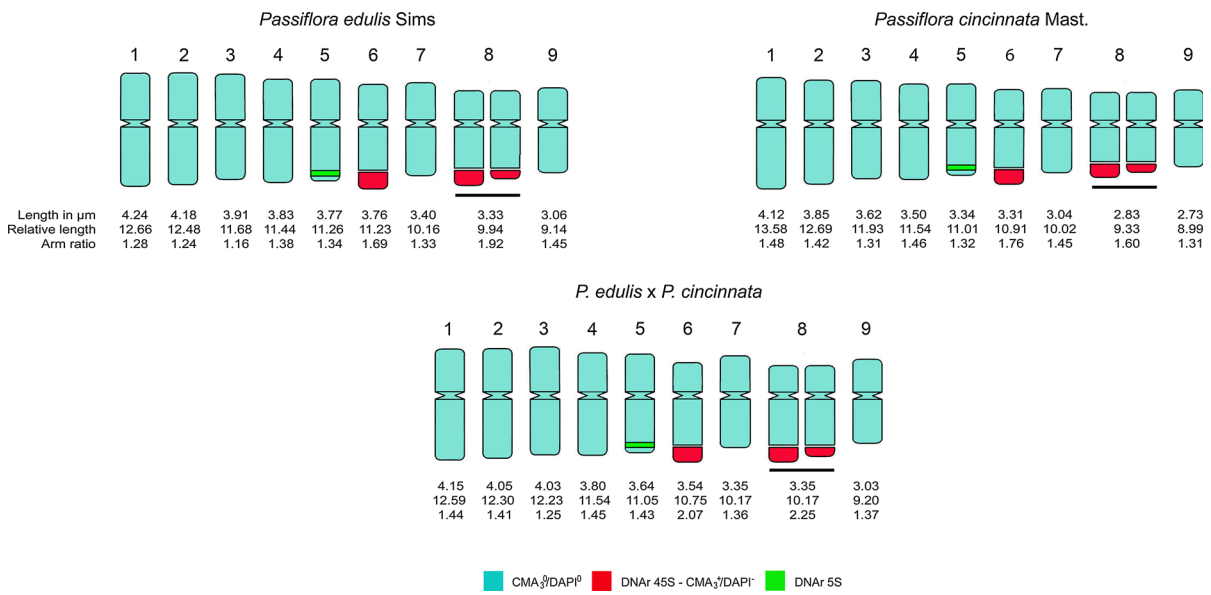

Fig. 3 Ideograms for *P. edulis, P. cincinnata* and *P. edulis* × *P. cincinnata* hybrid. The chromosomes were aligned according to the size in decreasing order and identified by numbers. The 5S rDNA site is on the long arm of chromosome 5. The CMA_3^+ bands and 45S rDNA sites are co-localized on the long arm of chromosomes 6 and 8. For the chromosome 8 that presented band size heteromorphism, two homologues were represented

Amplification of DNA

The 20 ISSR primers used to amplify genetic material from *Passiflora* species and the interspecific hybrid, and the characteristics of amplicons are provided in Table 2. The PCR amplifications were completed on PCR plates in a final volume of 15 µL, which consisted of 20 ng DNA, 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 0.4 µM primers, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, and 0.5 U *Taq* DNA polymerase. The PCR program was as follows: 94 °C for 3 min; 39 cycles of 94 °C for 45 s, 50 °C for 1 min, and 72 °C for 1 min; 72 °C for 7 min. Amplicons were analyzed by 1.5 % (w/v) agarose gel electrophoresis at a voltage of 80 V for 170 min. Gels were stained with ethidium bromide. The 1 kb DNA ladder (Affymetrix) was used as the molecular mass standard. After electrophoresis, DNA bands were visualized by exposure to ultraviolet light, and images were captured using LPix-STi photo documentation equipment. The PCR products were examined for the presence (1) and absence (0) of bands.

Results

Analysis of meiosis

Regular meiotic behavior was observed in all interspecific hybrid samples (*P. edulis* × *P. cincinnata*) and the parents. Additionally, nine bivalents were detected during the diplotene and/or diakinesis stages (Fig. 1a). This resulted in regular chromosomal segregations during anaphase I (Fig. 1b) and anaphase II (Fig. 1d) without the formation of anaphasic bridges. However, in the hybrid, some bivalents exhibited partial pairing, indicating the presence of small homologous regions (arrows in Fig. 1a).The meiotic regularity of the parents indicated their pollen grains were mostly viable (Table 1), suggesting they were suitable for assisted crosses. Additionally, the diameter of viable pollen grains was larger than that of non-viable grains (Table 1; Fig. 1e, f).

Analysis of mitosis

The karyotype analysis results revealed similar characteristics between the parents and hybrid, including the presence of semi-reticular interphasic nuclei (data not shown), and an identical diploid chromosome number ($2n = 18$) (Fig. 2). The absolute length of chromosomes was 4.24–3.06, 4.12–2.73, and 4.15–3.03 µm for *P. edulis*, *P. cincinnata*, and the interspecific hybrid, respectively. There were also 14 metacentric chromosomes and four submetacentric chromosomes in the three examined materials, indicating symmetry among the karyotypes (Fig. 3). The average and total chromosome lengths were highest for *P. edulis* (3.72 and 33.48 µm, respectively), followed by the interspecific hybrid (3.66 and 32.94 µm, respectively), and *P. cincinnata* (3.37 and 30.34 µm, respectively).

Double staining with CMA_3/DAPI revealed the presence of four CMA_3^+/$DAPI^-$ bands on the long arms of chromosome pairs 6 and 8 of *P. cincinnata* (Figs. 2a, 3), *P. edulis* (Figs. 2b, 3), and the hybrid (Figs. 2c, 3), which co-localized with the 45S rDNA sites detected by FISH (Fig. 2d–f). However, the smaller chromosome pair (i.e., pair 8) was heteromorphic regarding the size of the heterochromatic block. Additionally, two 5S rDNA sites were identified in the subterminal regions of the long arm of chromosome 5 for all examined genotypes (Figs. 2d, f, 3).

During the mitotic metaphases of the hybrid, the GISH results produced with the *P. edulis* probe revealed labeling differences, which enabled the identification of three chromosomal groups. The first group consisted of eight completely labeled chromosomes (Fig. $2j_1$), possibly originating from *P. edulis*. The second group was characterized by the presence of six partially labeled chromosomes (Fig. $2j_2$). The final group included four non-labeled chromosomes (Fig. $2j_3$) that likely originated from *P. cincinnata*, considering the genomic probe for this species hybridized with four chromosomes from the hybrid species (Fig. $2n_1$). Additionally, the *P. cincinnata* probe partially hybridized with six chromosomes from the hybrid plants (Fig. $2n_2$), similarly to what was revealed by the labeling with *P. edulis*, as exemplified by the chromosomes 6 and 8 with rDNA sites. This is also consistent with the findings for the diplotene stage of the hybrid plants, in which some chromosomes exhibited partial homology (arrows in Fig. 1a), while others displayed complete pairing. It is important to note that when the *P. cincinnata* probe was used to analyze *P. edulis* metaphases (with *P. edulis* DNA used as the blocker), no chromosomal labeling was observed (data not shown).

Table 2 ISSR primers used in the amplification of *Passiflora* L. species and an interspecific hybrid with their respective sequences, total number of bands (NTB), number of polymorphic bands (NPB), percentage of polymorphism (P %), number of bands per genotype (NBG) and fragment amplitude (FA)

Primer	Sequence[a]	NTB	NBP	P (%)	NBG			AF (pb)
					P. cincinnata	*P. edulis*	Pe × Pc	
1. DiGA3′C	GAGAGAGAGAGAGAGAC	12	6	50.0	4	3	5	400–1250
2. DiGT5′CR	CRGTGTGTGTGTGTGTGT	17	5	29.4	7	5	5	500–1500
3. DiGT5′CY	CYGTGTGTGTGTGTGTGT	3	3	100.0	–	1	2	500–1000
4. TriCAC3′YC	CACCACCACCACCACYC	10	7	70.0	3	5	2	600–1200
5. TriCAC5′CR	CRCACCACCACCACCAC	8	5	62.5	2	3	3	600–1500
6. TriCAC5′CY	CYCACCACCACCACCAC	–	–	–	–	–	–	–
7. TriCAG	CAGCAGCAGCAGCAG	2	2	100.0	2	–	–	550–1000
8. TriCAG3′RC	CAGCAGCAGCAGCAGRC	–	–	–	–	–	–	–
9. TriCAG3′YC	CAGCAGCAGCAGCAGYC	12	6	50.0	3	5	4	600–1250
10. TriGTG3′RC	GTGGTGGTGGTGGTGRC	6	6	100.0	–	3	3	600–900
11. TriTGT	TGTTGTTGTTGTTGT	4	4	100.0	1	1	2	550–1500
12. TriAAC 3′RC	AACAACAACAACAACRC	9	6	66.7	1	4	4	300–1100
13. TriACA 3′RC	ACAACAACAACAACARC	9	6	66.7	2	3	4	350–1100
14. TriACT 3′RC	ACTACTACTACTACTRC	–	–	–	–	–	–	–
15. TriACG 3′RC	ACGACGACGACGACGRC	7	4	57.1	1	3	3	500–1200
16. TriTCG 3′RC	TCGTCGTCGTCGTCGRC	–	–	–	–	–	–	–
17. DiGA5′CR	CRGAGAGAGAGAGAGAGA	10	7	70.0	3	4	3	250–550
18. TriAGT 3′RC	AGTAGTAGTAGTAGTRC	–	–	–	–	–	–	–
19. TriGTA 3′RC	GTAGTAGTAGTAGTARC	4	4	100.0	4	–	–	350–950
20. TriGCA 3′RC	GCAGCAGCAGCAGCARC	12	12	100.0	9	3	–	350–1500
Average		8.33	5.53	74.8				

[a] R = A + G; Y = C + T

Inter-simple sequence repeat markers

We used 15 of the 20 tested ISSR primers (Table 2), resulting in the amplification of 125 fragments with sizes ranging from 250 to 1500 bp (average of 8.33 bands). Eighty-three fragments were polymorphic (average of 5.53 bands), which corresponded to an average polymorphism rate of 74.8 % (Table 2). The number of amplified fragments ranged from two to 17 (for the TriCAG and DiGT5′CR primers, respectively). All fragments amplified by TriCAG were polymorphic, which was similar to the results for the DiGT5′CY, TriGTG3′RC, TriTGT, TriGTA3′RC, and TriGCA3′RC primers. In contrast, the DiGT5′CR primer only amplified 29.4 % of the polymorphic fragments (Table 2).

Each genotype produced about the same number of bands, with 43 bands for *P. edulis* (34.4 %), 42 bands for *P. cincinnata* (33.6 %), and 40 bands for the interspecific hybrid (32 %). However, three bands were specific for *P. edulis*, 20 were specific for *P. cincinnata*, and four were specific for the interspecific hybrid (Table 2). The primers used in this study enabled the identification of the parental origins for the fragments in the interspecific hybrid, with two diagnostic bands verified for *P. cincinnata* and 20 bands verified for *P. edulis*, as indicated by the amplification products for the DiGA3′C primer (Fig. 4).

Discussion

Interspecific hybridizations may be useful for genetically improving important crop species because they allow the transfer of phenotypic characteristics and genetic adaptations (Slotte et al. 2008; Jorgensen et al. 2011). The hybridizations have a fundamental role in diversifying plant species. In the present study, the

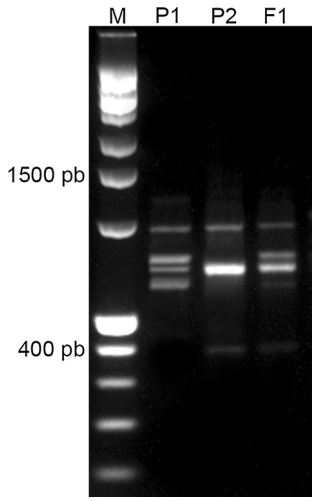

Fig. 4 ISSR profile using the primer DiGA3′C. The F1 hybrid present bands from both parents. *M* molecular mass marker (1 kb DNA ladder), *P1 P. cincinnata* (male parent), *P2 P. edulis* (female parent), *F1* interspecific hybrid

genomic contents of an interspecific hybrid and its parental *Passiflora* species were examined using cytogenetic and molecular techniques to identify potentially useful genotypes for the genetic improvement of passion fruits. Cytogenetic analyses indicated the absence of meiotic and mitotic irregularities in *P. cincinnata*, *P. edulis*, and interspecific hybrid plants. We observed the formation of bivalents and normal chromosomal segregation during anaphase I, the development of viable pollen grains, and the production of an F_2 generation. These findings suggested the hybrid was fertile. Additionally, no chromosomal variations were detected, as would be expected if there was a strong postzygotic barrier and genetic incompatibility (Moraes et al. 2013). These results implied the genomes of the analyzed plant materials were similar.

Most *Passiflora* species are diploid and are classified into groups according to the number of chromosomes, the presence of $CMA_3^+/DAPI^-$ bands, and the number of 45S rDNA sites (Melo and Guerra 2003). Thus, considering our results, the parental accessions *P. cincinnata* and *P. edulis* were classified into a group consisting of plants with a haploid chromosome number of $n = 9$ and four 45S rDNA sites that co-localize with $CMA_3^+/DAPI^-$ bands. This is consistent with the findings of published studies (Melo et al. 2001; Melo and Guerra 2003). However, the bands produced in this study were localized to chromosome pairs 6 and 8 in *P. cincinnata* and *P. edulis*, instead of to chromosome pairs 4 and 6 (*P. cincinnata*) and 7 and 9 (*P. edulis*) (Melo et al. 2001; Melo and Guerra 2003). These discrepancies may be due to differences in how the chromosomes were analyzed.

Similar to the parents, the sexual hybrid had a stable diploid chromosome number ($2n = 18$), probably because of the existing compatibility between the parents. This was also observed in the *P. edulis* × *P. setacea* interspecific hybrid (Soares-Scott et al. 2003). However, the chromosomal stability in hybrids is not always maintained, especially if post-hybridization intergenomic conflicts occur, which can lead to structural rearrangements, such as the elimination of chromosomes and alterations of the mitotic cycle. This situation has been reported for hybrids resulting from a *P. sublanceolata* ($2n = 22$) × *P. foetida* var. *foetida* ($2n = 22$) cross, which exhibited aneuploidy ($2n = 22 - 2$) likely because of chromosomal inactivation by nucleases, chromatin degradation, suppression of the centromere function, and asynchrony of the cellular cycle phases (Santos et al. 2012). It is noteworthy that the diploid chromosome number for *P. foetida* was previously reported to be $2n = 20$ (Melo et al. 2001; Melo and Guerra 2003). This may have been because of a counting error due to distended proximal chromosomal constriction or a tendency for karyotypes of hybrids to stabilize with a diploid chromosome number of $2n = 20$.

The hybrid produced the same number of CMA_3^+ bands and 45S rDNA sites as the parents. These markers were localized on chromosome pairs 6 and 8. In somatic hybrids or in parents with different ploidy levels, there is no reduction in the number of 45S rDNA sites in *Passiflora* (Cuco et al. 2005) and *Epidendrum* (Moraes et al. 2013) species. This fact may be related to the dynamic reorganization of repetitive DNA during the diploidization process, which occurs soon after a hybridization event (Leitch and Leitch 2008).

Our analyses of a chromosome pair bearing the 45S rDNA site ($CMA_3^+/DAPI^-$) revealed for the first time differences in the size of *Passiflora* 45S rDNA. This chromosomal heterozygosity may be the result of deletion events and/or amplification of the heterochromatin, which alters the length of the rDNA repetitions by repairing the mechanisms underlying concerted evolution, unequal crossing over, replication slippage, and/or translocation events (Eickbush and Eickbush

2007; Bhargava and Fuentes 2009; Mehrotra and Goyal 2014). Such mechanisms are responsible for the evolutionary dynamics of this type of chromatin, and may lead to chromosomal rearrangements in different plants, such as *Maxillaria* R.P. (Cabral et al. 2006) and *Vigna* Savi (Bortoleti et al. 2012) species.

In passion fruit, the possibility of genetic rearrangements between parents and their own hybrids (i.e., backcrossing) has been explored. Such rearrangements have been routinely examined by GISH because this technique examines the origin of various hybrids, enabling the detection of the introgression of genetic materials from parents (Raina and Rani 2001; Silva and Souza 2013), as observed in *Spondias* L. (Almeida et al. 2007), *Emilia* Cass. (Moraes and Guerra 2010), and *Passiflora* (Melo et al. 2015) species.

In the present study, GISH was used to differentiate among eight *P. edulis* chromosomes, suggesting a considerable incorporation of genomic sequences from this parent in the hybrid. However, we cannot confirm the occurrence of asymmetrical introgression in the hybrid because of the six detected partially labeled chromosomes. These partially labeled chromosomes may be used to reveal similarities in the genomes of species used for interspecific crosses (Risso-Pascotto et al. 2005). This is supported by the complete pairing of some chromosomes in the analyzed sexual hybrid, which favors the occurrence of genetic recombinations and regular chromosomal segregations. Consequently, fertile individuals are generated, as observed in the current study and previous studies involving *Passiflora* species (e.g., *P. sublanceolata* and *P. foetida* var. *foetida*) (Santos et al. 2012). The similarities among genomes may be because of the presence of retro-elements and other repetitive DNA fragments, which represent 19.6 % of the *P. edulis* genome (Santos et al. 2014).

The reproductive success of passion fruit hybrids may also be related to the auto-incompatibility system in allogamous species. This system favors cross-pollination and gene flow among genotypes (Ganga et al. 2004), which results in a high rate of genetic polymorphisms. The use of 7–30 ISSR primers can generate 50–200 polymorphic bands, which are sufficient to estimate the genetic relationships within and between species (Colombo et al. 1998). Results for the ISSR markers used in this study indicated there was a considerable abundance of genetic polymorphisms between the parental genotypes and the analyzed hybrid.

Our ISSR markers revealed the degree of relatedness between the studied genotypes by determining the parental origins of the hybrid fragments. The four and 20 diagnostic bands detected for *P. cincinnata* and *P. edulis*, respectively, confirm the introgression of genes from these species in the interspecific hybrid. This finding is consistent with those reported for *P. edulis* and *P. alata* (Santos et al. 2011; Costa et al. 2012) and other plants, including *Psammochloa villosa* (Poaceae) (Li and Ge 2001) and *Vitis* L. species (Wu et al. 2009). The results indicate these crossings may be relevant for crop genetic improvement programs (Melo et al. 2015). Our analyses confirm the genetic origins of the hybrid produced from the *P. edulis* × *P. cincinnata* cross. The hybrid is fertile and may be useful for retro-crosses with cultivated species or for creating a new genetic resource for the improvement of passion fruits.

Conclusions

- The *P. edulis* × *P. cincinnata* interspecific hybrid is fertile, and may be used to breed new passion fruit cultivars.
- The GISH results confirmed the genetic origins of the generated hybrid, and enabled the differentiation between the hybrid and parental genomes. Furthermore, GISH may be useful for identifying *Passiflora* interspecific hybrids. This technology represents a quick and reliable method to study the evolution of passion fruits, and may be relevant for breeding programs.
- The selected ISSR markers are promising tools for detecting polymorphisms in passion fruit genotypes, and for determining the possible relationships between genotypes. These markers may be useful for characterizing BAG-passion fruits and for genetic pre-breeding.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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