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Establishment of the genomic *in situ* hybridization (GISH) technique for analysis in interspecific hybrids of *Passiflora*

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ABSTRACT. The genomic *in situ* hybridization (GISH) technique was applied to *Passiflora* interspecific F_1 HD13-133 hybrids (*Passiflora sublanceolata* x *Passiflora foetida*) and HD15-101 (*Passiflora gardineri* x *Passiflora gibertii*), and the backcrossed hybrids (BC_1) HD18-106 and HD18-113 (*Passiflora sublanceolata* x HD13-133). GISH was performed using genomic probes prepared with the DNA from the paternal genitor, whereas the maternal DNA was used as blocking DNA and employed at various concentrations (20X, 40X, 60X, and 100X) in relation to the probe concentration. At the same time, GISH was applied with the use of simultaneous probes from both genomes, paternal and maternal, that were detected with avidin-FITC and anti-digoxigenin-rhodamine, respectively. Both methodologies allowed the distinguishing of the maternal and paternal genomes, thus confirming the hybrid nature of all the analyzed genotypes. Furthermore, the presence of recombinant chromosomes in BC_1 hybrids revealed the occurrence of meiotic recombination in HD13 hybrids. This application

of the GISH technique is an important step towards genomic analyses of *Passiflora* hybrids: it can broaden the phylogenetic and evolutionary studies of the genus and, at the same time, contribute to breeding programs.

Key words: Cytogenetics; Blocking DNA; Plant breeding; Backcrosses; Multicolor genomic *in situ* hybridization; Recombinant chromosomes

INTRODUCTION

Genomic *in situ* hybridization (GISH) is an efficient methodology for genomic analysis that is based on the molecular cytogenetics technique of fluorescence *in situ* hybridization (FISH) (Schwarzacher, 2003; Wang et al., 2009). Both techniques adopt probes for hybridization and detection of the target DNA; yet, GISH uses genomic probes that are prepared by cleavage and fragmentation of genomic DNA, usually from a parent hybrid, allowing the *in situ* detection of the genome that is homologous to the used probe (Stace and Bailey, 1999; Schwarzacher, 2003). GISH has been widely used in genomic studies of interspecific and intergeneric hybrids, enabling both the identification of parental genomes in natural and artificial hybrids and the identification of allopolyploids through the visualization of chromosomes derived from genitor species (Guerra, 2004; Lim et al., 2004). Likewise, inferences about genomic similarities amongst closely related or related species can be performed with the application of GISH, thereby making evolutionary and phylogenetic studies possible (Lim et al., 2000b). The application of GISH in plant breeding programs has made the study of chromosomal introgression possible once it exhibits, for example, the karyotype formation of backcrossed hybrids (Jahier et al., 2009).

The major difficulty in the application of GISH is the establishment and adjustment of the technique for the species or hybrid under study. This difficulty lies in adjusting the optimal concentration of blocking DNA in such a way to enable genomic distinction without nonspecific hybridization. In GISH, the blocking DNA should preferably consist of non-target genome; thus, the blocking DNA will compete with the genomic probes, enabling specific hybridization with the target DNA such that only the genome of interest is visualized (Tang et al., 2011; Brammer et al., 2013). The use of higher concentrations of blocking DNA relative to the concentration of genomic probes is usually required in interspecific hybrids derived from phylogenetically close genitors or those with similar large genomic sequences and consequently higher homology with the probes used. Low concentrations of blocking DNA, in turn, can be used in the application of GISH amongst interspecific hybrids whose parents exhibit greater genetic distance (Anamthawat-Jónsson et al., 1990).

Passiflora is a well-known genus that includes a number of fruit (*P. edulis* Sims and *P. alata* Curtis) and medicinal species (*P. foetida* L.) of economic importance (Puricelli et al., 2003). Another explored market is that of ornamental plants, which is represented by species like *Passiflora caerulea* L. (Abreu et al., 2009) and *Passiflora incarnata* L. (Rushing, 2003), as well as interspecific hybrids that are generally the result of crossings between wild species (Abreu et al., 2009) such as *Passiflora* 'Sunburst' (Vanderplank, 2000). The use of passion fruits as ornamental plants is justified by the great beauty of their exotic flowers. In Europe and the United States, they are already used for decoration (Rushing, 2003). Although Brazil has great potential for the use of these passion fruits as ornamental plants and the optimum

climatic conditions for their cultivation, this market niche remains virtually untapped in the country (Abreu et al., 2009; Santos et al., 2012).

In Brazil, *Passiflora* breeding programs and the production of interspecific hybrids have been conducted with the purpose of obtaining ornamental plants with unique features that are chiefly focused on the edaphoclimatic conditions. To that end, hybrids involving the species *P. sublanceolata* and *P. foetida*, which belong to the subgenus *Dysosmia*, supersection *Stipulata*, and section *Dysosmia*, and *P. gardneri* and *P. gibertii*, which belong to the subgenus *Passiflora*, supersection *Stipulata*, and section *Granadillastrum* (Ulmer and MacDougal, 2004) have already been produced. Phylogenetic studies based on plastid sequences and DNA content have revealed great genetic similarity between *P. sublanceolata* and *P. foetida*. Nonetheless, there is lower genetic similarity between *P. gardneri* and *P. gibertii* (Yotoko et al., 2011). Hybrids obtained from *P. sublanceolata* x *P. foetida* (Santos et al., 2012) and *P. gardineri* x *P. gibertii* (Belo, 2010) were recorded by the *Passiflora* Society International (http://www.passiflorasociety.org) as *Passiflora* 'Alva' (reference code #120 in 2008), *Passiflora* 'Aninha' (#121 in 2008), *Passiflora* 'Priscilla' (#122 in 2008), *Passiflora* 'Gabriela' (#170 in 2010), and *Passiflora* 'Bella' (#171 in 2010). Backcrosses (BC_1) have been conducted in hybrids of the progeny with the maternal parent *P. sublanceolata* (Moura et al., 2013).

Karyotypic studies involving the genus *Passiflora* point to the existence of chromosomal variation, suggesting that the basic number of chromosomes can be either $x_1 = 6$ or $x_1 = 12$, and $x_2 = 9$ is merely an important secondary basic number; moreover, diploid and polyploid may be the primary cytoevolutionary routes of the group (De Melo et al., 2001). In regard to the genus *Passiflora*, the use of molecular cytogenetic techniques has been rarely reported, whereas many of the existing studies were concerned with the distribution of the 45S and 5S ribosomal sites (De Melo and Guerra, 2003; Souza et al., 2008; Viana and Souza, 2012). The application of GISH in *Passiflora* has not been reported in the literature, limiting the knowledge of genomic relationships between the species and the interspecific hybrids.

This study aimed to establish the GISH technique and its application amongst interspecific *Passiflora* hybrids. The aim was therefore to combine cytogenetic and genomic studies of the genus, identify artificial and natural interspecific hybrids, and infer the participation of parental genomes in the karyotypic constitution of interspecific hybrids.

MATERIAL AND METHODS

Plant material

The plant material used for GISH was granted by wild passion fruit breeding programs that were directed at obtaining hybrids with ornamental potential (Table 1). The abbreviation HD refers to the progeny, whereas the following number refers to the hybrid.

Slide preparation

Root tips from cuttings were pre-treated with 0.002 M 8-hydroxyquinoline solution at room temperature (RT) for 1 h and stored for 21 h at 8°C until use. Fixed roots tips were washed twice with distilled water for 5 min, fixed in freshly prepared Carnoy fixative (3:1

ethanol:acetic acid [v/v]; Johansen, 1940) for at least 3 h at RT, and stored in a freezer at -20ºC until use. For slide preparation, radicles were washed twice with distilled water and incubated in an enzymatic solution of 2% cellulose and 20% pectinase (w/v) for 60-80 min at 37°C. Roots tips were washed with distilled water and macerated on a slide with a drop of 45% acetic acid, and a coverslip was added to the material and pressed. The coverslip was removed after freezing in liquid nitrogen, and the slide was air-dried and stored at -20°C until the GISH procedure.

Table 1. *Passiflora* interspecific hybrids and genitors used in hybridization, type of crossing, and chromosome number (2*n*) of genotypes used in genomic *in situ* hybridization.

Hybrids	Interspecific crossing and backcrossing	Type of crossing	2*n*
HD15-101 (Belo, 2010)	*P. gardinerii* x *P. gibertii*	F_1	18
HD13-133 (Santos et al., 2012)	*P. sublanceolata* x *P. foetida*	F_1	22
HD18-106 (Moura et al., 2013)	*P. sublanceolata* x HD13-133	BC_1	22
HD18-113 (Moura et al., 2013)	*P. sublanceolata* x HD13-133	BC_1	22

F_1 = crossing, first generation; BC_1 = backcrossing, first generation.

Preparation of genomic probes and cleavage of blocking DNA

Genomic probes were prepared using the genomic DNA of the paternal genitor from each interspecific hybrid: *Passiflora gibertii* for the hybrid HD15-101, *P. foetida* for the hybrid HD13-133, and HD13-133 for the hybrids HD18-106 and 113. Young leaves were extracted using the protocol proposed by Doyle and Doyle (1990) and quantified by electrophoresis on a 1% agarose gel using SYBR safe (Invitrogen, United States, S33102) as a nucleic acid dye. To prepare the probes, about 20 µg genomic DNA in a final volume of 200 µL was cleaved using a sonicator (Qsonica, Q125) with the following settings: 40% amplitude, 2 s on and 2 s off in a total period of 5 min (Jauhar and Peterson, 2006). The size of cleaved fragments was determined by electrophoresis on a 2% agarose gel (Pronadisa, 8012) using a 100-bp ladder marker (New England Biolabs, United States). After genomic DNA cleavage, purification was performed by precipitating the cleaved DNA with 2% of the final volume of 3 M sodium acetate plus 200% of the final volume of cold anhydrous ethanol. The mixture was stored at -20°C overnight and then centrifuged for 20 min at 14,000 rpm at 20°C to remove the supernatant. Next, the mixture was dried at RT for at least 1 h. The pellet was suspended in 10 mM Tris, pH 8.0, with the volume required for a final DNA concentration of 1.1 µg/µL. Probes were labeled by nick translation either with biotin-16-dUTP (Roche Diagnostics, United States, 11093070910) or digoxigenin-11-dUTP (Roche Diagnostics, United States, 11209256910) at a final concentration of 1 µg of cleaved DNA as proposed by the manufacturer protocol.

Blocking DNA was prepared using about 30 µg genomic DNA from the maternal genitor at a minimum final volume of 300 µL, which was cleaved using three methods: (*i*) sonication with the same settings as the aforementioned preparation of genomic probes; (*ii*) autoclaving at 120°C for 5 min (Brammer et al., 2013); and (*iii*) incubation in a thermocycler (Eppendorf, Mastercycler) at 94°C for 15 min followed by ice for 10 min (Guerra, 2012). After verification of DNA cleavage on a 2% agarose gel, the cleaved DNA was purified following the same protocol that was used to prepare the probes.

GISH

GISH application followed the protocols for *in situ* hybridization that were proposed by Schwarzacher and Haslop-Harrison (2000) and Souza et al. (2010) with adaptations. Slides containing the cytological preparations were oven-dried at 37°C for at least 1 h and were then subjected to treatment with 100 µg/mL RNase (Sigma, United States, R5125) in 2X SSC buffer [0.3 M sodium chloride (Sigma, United States, S3014) and 0.03 M sodium citrate (Sigma, United States, S5941)]; the slides were then incubated in a humid chamber for 1 h at 37°C. The slides were twice immersed in 2X SSC at RT for 5 min and were then treated with 50 µL 10 mM HCl for 5 min. Next, slides were treated with 50 µL pepsin (Sigma, United States, P6887) [10 mg/mL pepsin and 10 mM HCl (1:100 v/v)]. The following washing stages were carried out in a stirring platform at 120 rpm (Biomixer, Mos-1). The slides were rinsed twice in 2X SSC at RT for 5 min, immersed in 4% paraformaldehyde for 10 min at RT, and washed twice in 2X SSC for 5 min. Cytological preparations were dehydrated in two steps: in 70% ethanol and in 96% ethanol, each for 5 min. After the slides were dried for 30 min at RT, the hybridization mixture was added at a final volume of 50% formamide (Sigma, United States, P9037), 10% dextran sulfate (Sigma, United States, 42867), 2X SSC, 0.13% sodium dodecyl sulfate (Bioagency, 161-0301N), and 33 ng genomic probe. Two tests regarding the blocking DNA were carried out: (*i*) using four concentrations of blocking DNA relative to the genomic probe concentration (33 ng): (a) 20X (660 ng); (b) 40X (1.36 µg) (only with the hybrid HD15-101), (c) 60X (1.98 µg) and (d) 100X (3.3 µg); and (*ii*) preparations without blocking DNA and using probes of both maternal and paternal genitors (only with hybrids HD15-101 and HD18-113).

The hybridization mixture was heated to 75°C for 10 min in a thermocycler and immediately transferred to ice for at least 5 min. Cytological preparations containing the hybridization mixture were denatured in a thermocycler containing a slide adapter (Techne, T-412a) at 75°C for 10 min and then were incubated overnight in a humidified chamber at 37°C. After hybridization, the slides were soaked in 2X SSC at RT for 5 min to remove coverslips and then were subjected to a post-hybridization bath at 42°C in a Dubnoff bath (Quimis, 9226ML). The bath consisted of two 5-min immersions in 2X SSC, another two 5-min immersions in 0.1X SSC, and two 5-min immersions in 2X SSC. The slides were dipped in a 4X SSC and 0.02% Tween 20 (Sigma, United States, P7949) solution at RT for 5 min and were treated with 5% bovine serum albumin (BSA), fraction V (Sigma, United States, A3059). Biotin-labeled genomic probes were detected with 0.7 µL avidin-fluorescein isothiocyanate (FITC) (Vector, United Kingdom, SA-5001) plus 19.3 µL 5% BSA per slide. For the second test (*ii*), biotin-labeled and digoxigenin-labeled genomic probes were detected with the addition of 0.7 µL avidin-FITC and 0.7 µL anti-digoxigenin-rhodamine (Roche, United States, 11207750910) plus 18.6 µL 5% BSA per slide. Slides containing the antibodies for detection were incubated in a humid chamber for 1 h at 37°C. To remove excess antibodies, three 5-min baths were carried out in 4X SSC and 0.2% Tween 20 at RT. Slides were briefly rinsed with 2X SSC, and cytological preparations were simultaneously counterstained and mounted with DAPI medium/Vectashield® (H-1200). The slides were stored for up to 3 days at 6°-8°C until analysis.

Photo-documentation and analysis

Slides were examined using an Olympus BX41 epifluorescence microscope fitted with a DP25 photo-documentation system. To visualize FITC and tetramethylrhodamine iso-

thiocyanate (TRITC) fluorochromes, the filters U-MWB (450-480 nm excitation, 500 nm dichroic cutoff, emission > 515 nm) and U-MWG (510-550 nm excitation, 570 nm dichroic cutoff, emission > 590 nm) were used, respectively, whereas the counterstaining with DAPI was visualized using the U-MWU filter (330-385 nm excitation, 400 nm dichroic cutoff, emission > 420 nm). Photo-documentation was performed by the DP25 5 Megapixel system with the DP2-BSW software (Olympus). Two image-capture methods were tested: (*i*) high exposure (> 2 s) with ISO 50 and (*ii*) low exposure (< 250 ms) with ISO 200. Analysis of results, preparation of figures, and FITC/DAPI overlays (superposition of layers using 75% opacity of FITC) were carried out using the Photoshop SC5 software (open source).

RESULTS

To prepare the blocking DNA for GISH, the genomic DNA of the paternal genitors, *P. gardneri* and *P. sublanceolata*, were cleaved using three different methods that aimed to obtain fragments between 100 and 800 bp (Figure 1). The use of a sonicator in the cleavage of genomic DNA resulted in the best cleavage profile after gel electrophoresis on 2% agarose. Sonication resulted in the cleavage of fragments greater than 200 bp but with a prevalence of fragments between 200 and 1000 bp. The use of an autoclave in the preparation of blocking DNA only cleaved genomic DNA to fragments smaller than 200 bp, and thermal shock produced high concentrations of fragments greater than 1200 bp. Therefore, blocking DNA for GISH was cleaved by sonication.

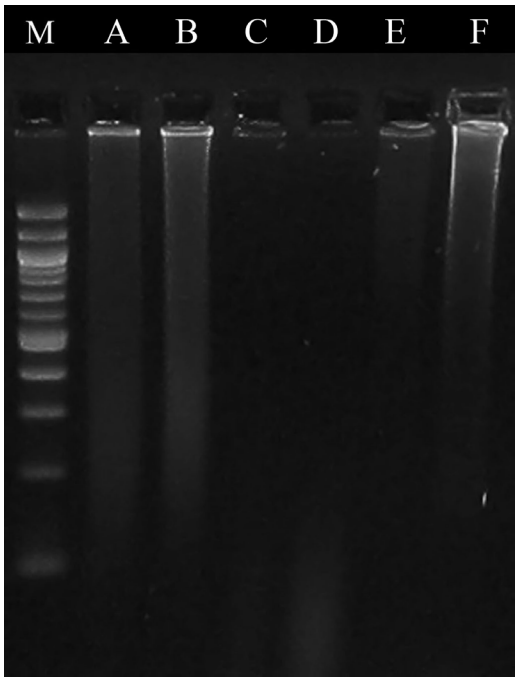

Figure 1. Agarose gel electrophoresis of 1.1 µg blocking DNA used for genomic *in situ* hybridization (GISH) in *Passiflora*. *Lane M* = 100-bp ladder; *lanes A* and *B* = cleavage using a sonicator; *lanes C* and *D* = cleavage using autoclave; *lanes E* and *F* = cleavage using thermal shock; A, C, and E, *P. sublanceolata*; B, D, and F, *P. gardneri*.

GISH of interspecific hybrids F_1 HD15-101 ($2n = 18$), HD13-133 ($2n = 22$), and BC_1 HD18-106 ($2n = 22$) using blocking DNA with a concentration 20X higher than that of the genomic probes of paternal genitors did not exhibit good results because of cross-hybridization, making it impossible to safely distinguish the target genome (Figure 2A-C). In analysis of the interspecific hybrid HD15-101, the use of 40X blocking DNA allowed the visualization of hybridized chromosomes, giving the target genome the typical green fluorescence (FITC); nevertheless, the strong occurrence of cross-hybridization with non-target genomes revealed the need of using blocking DNA (Figure 2D).

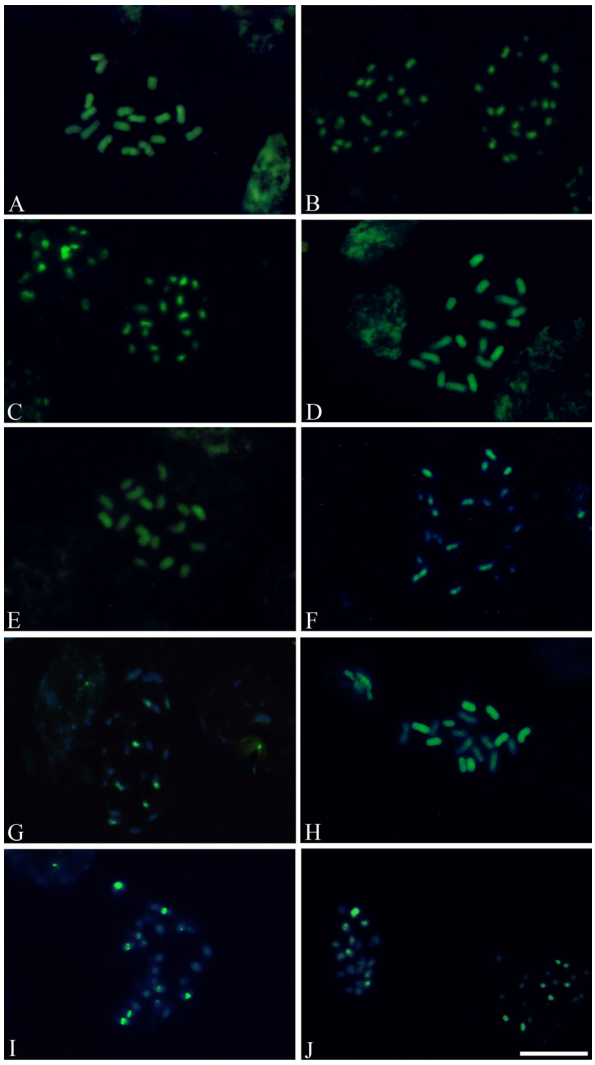

Figure 2. Application of GISH in interspecific and backcrossed hybrids of *Passiflora*. **A**, **D**, **E**, and **H**, interspecific hybrid F_1 HD15-101 with the following blocking DNA concentrations: 20X, 40X, 60X, and 100X, respectively; **B**, **F**, and **I**, interspecific hybrid HD13-133 with 20X, 60X, and 100X blocking DNA, respectively; **C**, **G**, and **J**, BC_1 HD18-106 with 20X, 60X, and 100X blocking DNA, respectively. Bar = 10 µm.

GISH using 60X blocking revealed the involvement of nine chromosomes of *P. gibertii*, which is the target genome, in the genomic constitution of HD15-101 (Figure 2E). Hence, the use of 60X blocking DNA is an option to distinguish hybrid genomes in the hybrids of the progeny HD15. Yet, chromosomes from the maternal genitor (*P. gardneri*) in HD15-101 also showed hybridization signals. The application of GISH with 60X blocking DNA in HD13-133 and HD18-106 showed specific hybridization with some chromosomes of the target genome, thus enabling the visualization of chromosomes derived from the paternal genitors (Figure 2F and G). On the other hand, chromosomes from the non-target maternal genome showed cross-hybridization (especially the larger chromosomes), leading us to question whether translocations could exist in the progeny HD18, especially when high exposures (> 2 s) or high ISO (200) are used in photo-documentation.

The use of 100X blocking DNA revealed the presence of nine strongly hybridized chromosomes in the hybrid HD15-101, representing the genome of *P. gibertii* in the genomic constitution of the hybrid (Figure 2H). In HD15-101, the nine chromosomes of the maternal genitor (*P. gardneri*), whose DNA was used as blocking DNA, showed no hybridization signals. This demonstrated that the use of 100X blocking DNA makes possible a clear distinction of the genitor genomes in HD15-101.

In HD13-133, the use of 100X blocking DNA resulted in specific hybridization of the target genome, as 12 chromosomes were partially or fully hybridized, allowing the safe distinction of both genomes in the constitution (Figure 2I). The use of 100X blocking DNA in the hybrid HD18-106 indicated the existence of three fully hybridized chromosomes and another five chromosomes with hybridization signals on the short or long chromosome arms. This revealed the presence of chromosomes with recombination between the maternal (*P. sublanceolata*) and the paternal genomes (*P. foetida*) in HD18-106 (Figure 2J).

The application of GISH using probes from both genitors simultaneously and without the addition of blocking DNA made the distinction of the genitor genomes possible. In the prometaphases and metaphases of the hybrid HD15-101, nine hybridized chromosomes with genome probes of the paternal genitor were observed, and they were detected with avidin-FITC (green) and visualized with a U-MWB filter. The same filter that was used to detect FITC signals excited TRITC fluorescence (red) and revealed nine hybridized chromosomes with the probes of the maternal genitor, which, due to the mixing of FITC-TRITC signals, showed an opaque fluorescence (Figure 3A and B). The use of a U-MWG filter to detect the maternal genomic probes (TRITC signals) in HD15-101 exhibited nine strongly hybridized chromosomes (Figure 3C).

In the hybrid HD13-133, the application of GISH with the use genomic probes from both parents simultaneously and without the addition of blocking DNA revealed 12 chromosomes with clear signals of paternal genome probe hybridization, which were detected with avidin-FITC/U-MWB, and another 10 chromosomes that were hybridized with probes of the maternal genome, which were detected with anti-digoxigenin-rhodamine and visualized using the same filter, revealing an opaque yellow staining as a result of the mixing of the FITC and TRITC signals (Figure 3D). In HD18-113, in turn, the application of GISH with the concomitant use of probes without blocking DNA revealed nine chromosomes that strongly hybridized with the paternal genomic probes, which were detected with a U-MWB filter for FITC fluorescence. One chromosome only exhibited a hybridization signal on one chromosome arm, denoting recombination (Figure 3E). With the aid of a U-MWB filter, chromosomes derived from the maternal genome in HD18-113 showed an opaque yellow fluorescence (Figure 3E).

The use of a U-MWG filter to detect TRITC fluorescence did not allow the indisputable distinction of maternal and paternal genomes in HD13-133 and HD18-113 (Figure 3F).

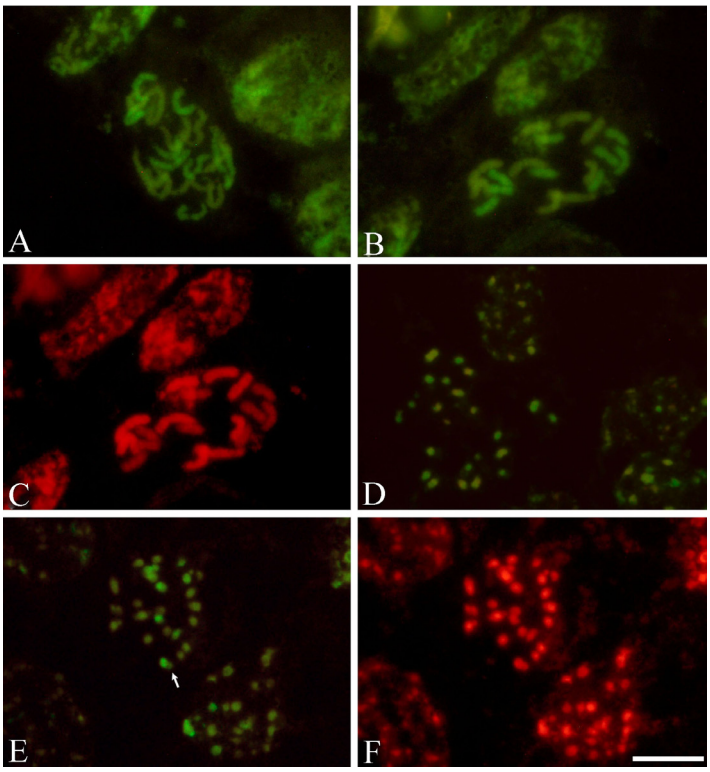

Figure 3. Two probes for GISH without blocking DNA in *Passiflora*. Paternal and maternal genomic probes were detected by avidin-fluorescein isothiocyanate (FITC, green) and anti-digoxigenin-rhodamine (red), respectively. **A** and **B**, prometaphase and metaphase chromosomes of the hybrid F_1 HD15-101 detected by a U-MWB filter showing nine chromosomes from each maternal (yellow) and paternal (green) genomes; **C**, maternal chromosomes of F_1 HD15-101 detected by anti-digoxigenin-rhodamine (red); **D**, metaphase chromosomes of the hybrid F_1 HD13-133 showing chromosomes from the maternal (yellow) and paternal (green) genomes; **E**, BC_1 HD18-113 detected by avidin-FITC (green) for paternal chromosomes and yellow for maternal chromosomes; **F**, BC_1 HD18-113 detected by anti-digoxigenin-rhodamine (red) where no genomic distinction can be made. Arrow in E indicates recombinant chromosomes in BC_1. Bar = 10 µm.

DISCUSSION

In this study, the GISH technique was first applied in *Passiflora* to distinguish the parental genomes in interspecific ornamental hybrids. Besides confirming the hybrid character, GISH allowed the observation of recombinant chromosomes in backcrossed hybrids (BC_1). The use of GISH to identify natural hybrids, to confirm artificial hybridization (Marasek et al., 2004; Contreras et al., 2012), and to identify recombined chromosomes in backcrossed progenies BC_1 and BC_2 (Karlov et al., 1999; Lim et al., 2000b; Marasek-Ciolakowska et al., 2012) has been widely reported for different plant groups. Hybrids BC_1 HD18-106 and HD18-113 showed different numbers of recombinant chromosomes, denoting variation in the meiotic

recombination amongst homeologous chromosomes in HD13 hybrids. A wide variation in the quantity of recombinant chromosomes is commonly reported in the hybrids BC_1 and BC_2 (Marasek-Ciolakowska et al., 2012). GISH is also used in the analysis of recombination in meiotic chromosomes (Lim et al., 2000a).

Establishing the GISH technique requires the adjustment and optimization of several factors, namely (*i*) the size of DNA fragments in the preparation of probes and/or as blocking DNA, (*ii*) use of probes from both genitors, (*iii*) variations in the levels of stringency used in post-hybridization baths, and (*iv*) variations in the concentrations of probes and blocking DNA added to the hybridization mix (Schwarzacher and Haslop-Harrison, 2000; Czernicka et al., 2010).

In order to perform GISH in *Passiflora* hybrids, the sonication of genomic DNA was the most efficient cleavage method amongst the methods tested. Sonication was seen to provide the best profile after electrophoresis, with a predominance of fragments ranging from 200 to 1000 bp, i.e., more suitable for the preparation of genomic probes and labeling more efficient by nick translation (Schwarzacher and Haslop-Harrison, 2000). Genomic DNA fragments ranging between 50-300 bp are commonly used to block the hybridization of repetitive DNA sequences present in the probe. Depending on the stringency used in hybridization, those fragments also block similar sequences from both genitor species of an interspecific hybrids (Wilkinson, 1998; Schwarzacher and Haslop-Harrison, 2000). In turn, larger fragments, between 1 and 10 kb, were used for the preparation of genomic probes directed to some genera, such as *Lilium* (Lim et al., 2004).

In this study, the DNA that was cleaved by sonication was both employed in the preparation of genomic probes and used as blocking DNA. Some methods proposed for GISH apply two different methods for the cleavage of genomic DNA: one directed to the cleavage of DNA for probes and another for blocking DNA, wherein sonication is generally used to cleave the DNA for the probe, and the autoclave is used to cleave the blocking DNA (Jauhar and Peterson, 2006; Marasek-Ciolakowska et al., 2012). Various reports on the use of autoclaving as a sole DNA cleavage method either in the preparation of probes or in the direct use as blocking DNA suggest that autoclaving is an efficient method of DNA fragmentation (Lim et al., 2004; Brammer et al., 2013). Nevertheless, as revealed in this study, small fragments (<100 bp) were obtained with the simple use of an autoclave for 5 min at 120°C, showing the need to adjust the DNA fragmentation method. DNA cleavage by autoclaving can be influenced by parameters such as temperature, fragmentation time, final volume, and autoclave model; there is a large variation in fragment sizes relative to the time of fragmentation (Jauhar and Peterson, 2006; Marasek-Ciolakowska et al., 2012; Brammer et al., 2013).

The best results were obtained when the concentrations of the probe and blocking DNA were 33.3 ng and 33.3 µg, respectively, per slide, revealing the use of 100X blocking DNA compared to the probe. Deviation from this optimum point (100X concentrated), both relative to the concentration of the probe and the proportion (probe:blocking DNA), influenced the resolution of results (Brammer et al., 2013). Using high concentrations of blocking DNA in *Passiflora* hybrids reveals a strong tendency towards cross-hybridization, which is usually caused by the similarities between repetitive DNA sequences that are common in the maternal and paternal genomes (Marasek et al., 2004). The addition of blocking DNA at concentrations higher than that of the probe is the most varied parameter amongst the several protocols that were proposed for GISH; it was adjusted as a function of the evolutionary and taxonomic levels of the genitor species. However, the greater the phylogenetic and genomic

proximity between the genitors, the higher are the concentrations of blocking DNA in order to reduce cross-hybridization (Schwarzacher and Haslop-Harrison, 2000).

The simultaneous addition of more than one probe to the hybridization mix is called the multicolor GISH (mGISH) technique (Li et al., 2001a,b; Xiong et al., 2006). Even at low stringency levels, mGISH showed good resolution in the karyotypic characterization of species of complex genomic constitution, such as allotetraploids (Li et al., 2001a,b). Cytological observations demonstrated that the simultaneous use of probes from both genitors of *Passiflora* hybrids enabled the visualization of both genomes among the hybrids that were analyzed in this study. The simultaneous use of two probes, one from each genitor, is not indicated in cases of genetically close genitors because high levels of cross-hybridization can lead to a mixture of both fluorescences. In these cases, the use of blocking DNA along with the two probes may be necessary (Schwarzacher and Haslop-Harrison, 2000).

Likewise, the use of only one filter (Olympus U-MWB) specific for FITC (green) detection made possible the visualization of chromosomes preferably hybridized by the probes detected by anti-digoxigenin-rhodamine, making clear the mixing of FITC-TRITC signals (opaque yellow). The ability of specific filters to detect FITC fluorescence and at the same time to excite red fluorescence (TRITC) was observed in *Passiflora*, where strong TRITC hybridization sites are seen in conjunction with FITC hybridization sites, and TRITC fluorescence becomes yellow (De Melo and Guerra, 2003). The inability of a U-MWG filter to detect the maternal genome using anti-digoxigenin-rhodamine (strong hybridization signal), as well as to visualize hybridization signals in hybrids HD13-133 and HD18-113, can be ascribed to the small size of chromosomes in conjunction with the genomic similarity between the genitors *P. sublanceolata* and *P. foetida* (Yotoko et al., 2011). On the other hand, the U-MWG filter made possible a clear distinction between the maternal and paternal genomes in HD15-101; this fact is related to the larger chromosome size and a greater genomic distance between the genitors. The application of GISH in hybrids or species with small chromosomes may lead to a loss of resolution in hybridizations, and the occurrence of hybridization sites might be limited to pericentromeric or heterochromatic regions (Czernicka et al., 2010; Marasek-Ciolakowska et al., 2012).

The application of GISH in *Passiflora* showed unambiguous and reliable results relative to the distinction of genitor genomes in hybrids. The results obtained by both methodologies, GISH using blocking DNA and GISH using two probes simultaneously, enlighten the research decisions about the most suitable technique for germplasm in analysis. Nonetheless, both techniques showed positive results even with small chromosomes (1 to 2.5 µm) when it came to the hybrids HD13-133, HD18-106, and HD18-113. GISH allowed the observation of recombination amongst hybrids derived from backcrosses (BC_1), which broadens the possibilities of genomic analysis in natural and artificial hybrids. Hence, the application of GISH in *Passiflora* enables the recognition of potential genitor species, and it can be used for cytogenetic analysis and detection of morphological markers in breeding programs.

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