CYTOGENETIC RELATIONSHIPS IN THREE VARIETIES OF *Tigridia pavonia* (L.f.) DC †

[RELACIONES CITOGENÉTICAS EN TRES VARIEDADES DE *Tigridia pavonia* (L.f.) DC]

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SUMMARY

Background: *Tigridia pavonia* (L.f.) DC is a wild species with great ornamental value, of which nine plant varieties are known. Within the evolutionary process of this species, Penélope has been considered a natural hybrid, product of the cross between the varieties Trinidad and Dulce. **Objective:** In the present study, the cytogenetic relationships among Trinidad, Dulce and Penélope were analyzed. **Methodology:** The karyotype of the varieties Trinidad and Penélope was determined through classic cytogenetics and the physical mapping of the genes 5s and 45s rDNA through Fluorescent *In Situ* Hybridization. **Results:** The results showed for the first time the karyotype and the physical mapping of the genes 5s and 45s rDNA in the varieties Trinidad and Penélope. **Implications:** The information generated can be the basis for future evolutionary analyzes, and / or breeding programs in the species. **Conclusion:** A higher cytogenetic similarity of Penélope with Trinidad and Dulce has been revealed, suggesting that the latter may be the parents.

Keywords: *Tigridia pavonia*; natural hybrid; karyotype; Fluorescent *In Situ* Hybridization; B chromosomes.

RESUMEN

Antecedentes: *Tigridia pavonia* (L.f.) DC. (Iridaceae) es una especie silvestre de gran valor ornamental, de la cual se conocen nueve variedades vegetales. Dentro del proceso evolutivo de la especie, Penélope ha sido considerada un hibrido natural producto de la cruza entre las variedades Trinidad y Dulce. **Objetivo:** En el presente estudio se analizaron las relaciones citogenéticas entre las variedades Trinidad, Dulce y Penélope. **Metodología:** El cariotipo de las variedades Trinidad y Pénelope se determinó mediante citogenética clásica, y el mapeo físico de los genes 5s y 45s rDNA a través de Hibridación Fluorescente *In Situ*. **Resultados:** Los resultados mostraron por primera vez el cariotipo y el mapeo físico de los genes 5s y 45s rDNA en las variedades Trinidad y Penélope. **Implicaciones:** La información generada puede servir de base para análisis evolutivos, y / o programas de mejoramiento genético en la especie. **Conclusión:** Se revelo una alta similitud citogenética de Penélope, con respecto a Trinidad y Dulce, sugiriendo que estos pueden ser los progenitores.

Palabras clave. *Tigridia pavonia*; híbridos naturales; cariotipo; Hibridación *In Situ* Fluorescente; cromosomas B.

INTRODUCTION

Tigridia pavonia (L.f.) DC. (Iridaceae) is a Mexican wild species of great ornamental value. Although in Mexico its flower has a historical and social importance, their diffusion and production are limited (Vázquez-García, 2011a; Peña Lomelí *et al*., 2013). Nowadays, of the 43 species of this genus reported in México only *T. pavonia* is commercialized in several countries (Vázquez-García, 2011a; Munguía-Lino *et al.*, 2015), being appreciated mainly as gardening plant (Carrillo-Ocampo *et al.*, 2002).

Cytogenetic analyzes are an important tool for the design of breeding programs, and for taxonomic and phylogenetic studies (Ramesh, 2015). In addition, with the development of the Fluorescent *In Situ* Hybridization (FISH) technique, it has been possible to characterize specific chromosomes, allowing the identification of chromosomal rearrangements and genomic changes between different groups species or

† *Submitted May 8, 2020 – Accepted July 15, 2020.*
ISSN: 1870-0462.

polyploid individuals (Guidini *et al*., 2017; He *et al.,* 2017).

Some studies have been focused at the genetic breeding of this species for example; Díaz-L *et al*. (2003) irradiated bulbs of the variety Sandra with gamma rays of ^{60}Co to induce variability of ornamental importance. Likewise, Piña-Escutia *et al.* (2003) obtained hybrids between *T. pavon*ia var Carolina and *T. augusta* by *in vitro* culture of ovary sections, which indicates an interest in the species.

The FISH technique has also been useful to confirms paternity in *Passiflora* hybrids (Silva *et al.*, 2018). In this regard, of the nine varieties of *T. pavonia* registered in 2016 in the Catálogo Nacional de Variedades Vegetales, it is considered that at least two of them could be natural hybrids, although so far no research has been reported that confirms it. Thus, it is believed that Penélope variety is one of them, whose parents could be Trinidad and Dulce varieties, the latter showing three B chromosomes in their karyotype (Arroyo-Martínez *et al.*, 2018). It is noteworthy pointed out that Penélope have showed intermediate features for color, height plant and fertility percentage in relation to Trinidad and Dulce varieties and the three share the same geographic location.

Karyotype characterization and the putative hybridization between these varieties becomes more relevant if B chromosomes effect is considered, because in the organisms that possess them, they could affect the biosynthesis of essential oils (*Salvia coccinea*); increases the esterase E-1 transcription (*Scilla autumnalis*); or decrease fertility (Oliver *et al*., 1982, Ghaffari and Bidmeshkipoor, 2002, Mani and Thoppil, 2005: Jones *et al.*, 2008). Thus, analysis of transmission of these structures would enhance strategies of genetic breeding focused on the fertility or production of seed of the *Tigridia* genus.

The aim of this study was to analyze the cytogenetic relationships among Trinidad, Dulce, and Penélope varieties through classical cytogenetic techniques and physical mapping of 5S and 45S rDNA genes, this may provide information that aid as a basis for determinate B chromosomes transmission as well as to elucidate the hybrid origin of *T. pavonia* var. Penélope.

MATERIALS AND METHODS

Plant material

The bulbs of Trinidad and Penélope varieties of *Tigridia pavonia* were donated by the Wild Species Conservation Center of the Tenancingo University Center, of the Universidad Autónoma del Estado de México (UAEMex). These were planted in pots with a substrate composed of soil, sand and cow manure (1: 1: 1) and maintained in a rustic greenhouse of the Facultad de Ciencias Agrícolas of the UAEMex until they flowering (Figure 1). Root meristems were collected for chromosomal studies.

Mitotic chromosomes observation

Slide preparations were performed in accordance with the methodology proposed by Barba-González *et al.* (2005). Briefly 15 metaphase cells from five plants of *T. pavonia* of each variety were observed. Radicular meristems were placed in a 0.002 M 8-hydroxyquinoline solution for 6 h at 4 °C under dark conditions. Subsequently, they were fixed in Farmer's solution for 24 h; and then treated with a mixture of enzymes at a final concentration of 0.2% (Cellulase, Pectolyase, Cytohelicase) in citrate buffer pH 4.5 for 2 h at 37 ° C, once the enzymatic digestion was completed, the meristem was placed in a slide and one drop of aceto-orcein (1%) was added. After placing the coverslips, the tissue was disintegrated and the cells were left in a single plane by the squash method, the preparations were made permanent by the liquid nitrogen method. The preparations were analyzed using an Olympus contrast phase microscope and the best cells of each species were photographed with a Leica MC170 HD camera.

Mitotic chromosome analyses and karyotype determination

The length of the chromosomal arms was measured by the LAZE V.4 software. Chromosomal morphology, total genome length in μm (LGT), and asymmetry index (TF%) were determined. Chromosomal morphology classification was carried out following the methodology proposed by Levan *et al.* (1964). Chromosome homology was established according to similarities in length and centromeric position. Idiograms were elaborated according to average values of the short arm and long arm in each pair of chromosomes and were grouped into metacentric (m), submetacentric (sm), subtelocentric (st) and telocentric (t) chromosomes. The asymmetry index (TF%) was obtained as reported by Sinha and Roy (1979).

Statistical analysis

In order to determine the possible significant differences of LGT and TF % between Trinidad and Penélope varieties, data were analyzed with a normality test. Likewise, an analysis of variance was carried out as well as a test of significant minimum difference (LSD) with software Statgraphics XV.I.

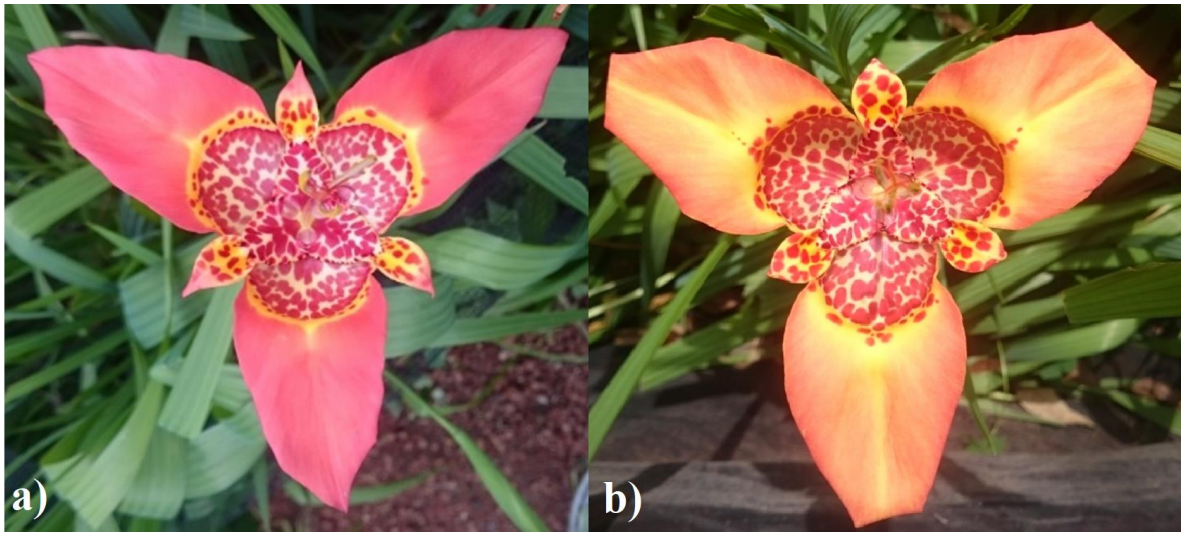

Figure 1. Flowers of two *Tigridia pavonia* varieties: a) Trinidad, b) Penélope.

Physical mapping of 45S and 5S rDNA genes

Physical mapping of 45S and 5S rDNA genes was carried out according to the methodology proposed by Barba-González *et al.* (2005) with some changes, briefly: for the slide preparation the root meristems were placed in a solution of 8-hydroxyquinoline 0.002 M for 6 h at 4 ° C in dark conditions. Subsequently, they were fixed in Farmer's solution for 24 h, and then incubated in a mixture of enzymatic digestion of pectolyase Y23 0.2% (w / v), cellulase RS 0.2% (w / v), and cytohelicase 0.2% (w / v) in citrate buffer 10 mmol / L (pH 4.5), at 37 ° C for approximately 2 h. The cell squash was made in a drop of 50% acetic acid and frozen in liquid nitrogen, the covers were removed with a razor blade and immediately dehydrated in 96% ethanol and air dried.

For Fluorescent *In Situ* Hybridization (FISH) technique, the 5S and 45S rDNA wheat genes were utilized as probes. Both were isolated with the High Pure Plasmid Isolation kit (Roche Diagnostics GmbH, Germany) and marked with Fluorescein-12-dUTP (Roche Diagnostics GmbH, Germany) and Tetramethyl-Rhodamine-5-dUTP (Roche Diagnostics GmbH, Germany), respectively by Nick Translation Mix, according to the manufacturer's instructions (Roche Diagnostics GmbH, Germany).

Probes hybridization was performed by incubating the slides in RNAse (100 µg / mL) for 1 h, and pepsin (5 µg / mL) for 10 min, both at 37 ° C, and later in paraformaldehyde (4%) for 10 min. at room temperature; They were then dehydrated with 70%, 90% and 100% ethanol for 3 min each, and air dried. Hybridization continued using a mixture of 20x SSC, formamide 50%, dextrasulfate sodium 10%, SDS 10%, and 25-50 ng / mL of each probe. The DNA was denatured by heating the hybridization mixture at 70 ° C for 10 min and placing it on ice for at least 10 min. For each slide, 40 µL of the hybridization mixture was used. The slides were denatured at 80 ° C for 10 min and then hybridized overnight at 37 ° C in a humid chamber. Subsequently, the slides were washed at room temperature in 2x SSC for 15 min and 0.1x SSC at 42 ° for 30 min. The chromosomes were counterstained with 1 µL / mL of DAPI (4 ', -diamidino-2-phenylindole), a drop of Vectashield antifade (Vector Laboratories) was added before examining the slides under a Leica DM3000 microscope (Leica Microsystems, Germany) equipped with epifluorescent lighting and coupled to a Leica DF200 camera (Media-Cybernetics, USA).

RESULTS AND DISCUSSION

Karyotype analyses

According to Arroyo-Martínez *et al.* (2018) each of the nine varieties of *T. pavonia* can present unique chromosomal features. In the present study it was observed that Trinidad and Penélope varieties presented significant differences in the karyotype, namely; Trinidad had a chromosome number of 2n = 2x = 28 (Figure 2) an LGT = 135.38 µm, with a size range of 8.83 to 11.32 µm for large chromosomes, and from 2.92 to 5.01 µm for small chromosomes. Also the presence of secondary constrictions in chromosomal pairs 3 and 6 was observed whereas karyotypic formula was 28m with a TF% of 46.16.

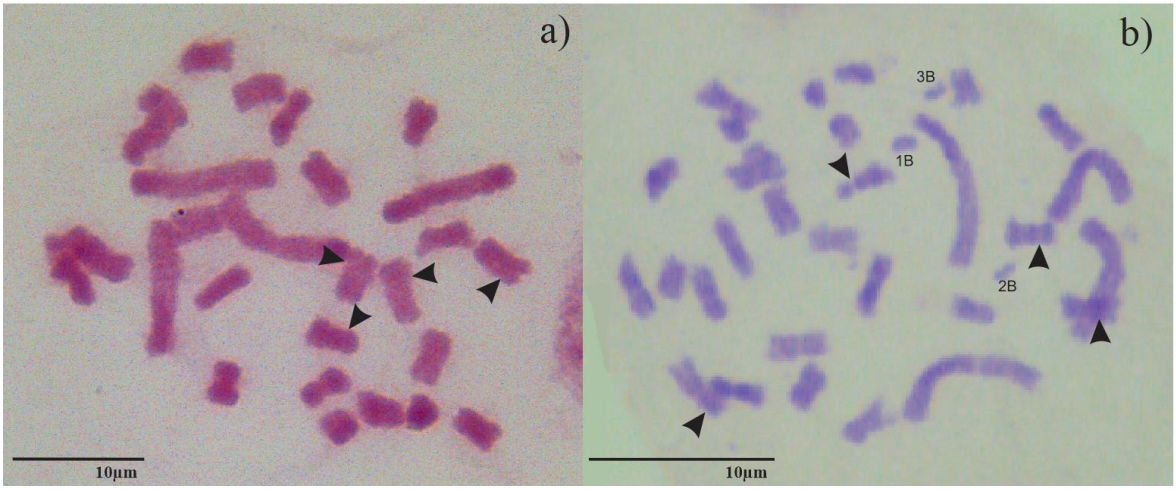

Figure 2. Chromosomes in mitotic metaphase of two *Tigridia pavonia* varieties: a) Trinidad showing 2n = 2x = 28, b) Penélope showing 2n = 2x = 28 + 3B. Arrowheads indicate the chromosomes with secondary constriction.

Our results are similar to the karyotype formula of Sandra variety (Arroyo-Martínez *et al.*, 2017) except to LGT as well as number and position of secondary constrictions. It is noteworthy that although both varieties have similar flower color, they come from distinct localities and their morphological characteristics as height plant, internal tepal shape and color disposition are also different (Vázquez-García *et al*., 2001b). In this sense Martinez *et al.* (2000) and Moreno Salazar *et al.* (2007) reported variations in the karyotype of populations of *Echeandia nana* and *Agave angustifolia* respectively, pointing out that geographic distance between populations besides physical barriers as hills and vegetation, are factors that prevent gene flow among them causing biogeographic or reproductive isolation and facilitating the differentiation of populations due to genetic drift. According to this it is possible that within differentiation process of *T. pavonia* varieties geographical distance has influenced the modification of the karyotype, originating specific characteristics of the karyotype of each variety.

On the other hand, Penélope presented a chromosome number of 2n = 2x = 28 + 3B (Figure 2), highlighting the three extra chromosomes. The karyotypic formula for this variety was of 26m + 2sm + 3B. Secondary constrictions were observed in chromosome pairs 5 and 7. The TF% was 46.16, and LGT = 116.55 µm; size range for large chromosomes was from 6.0 to 7.5 µm whereas for small chromosomes was from 4.35 to 2.92 µm, and for the three putative B chromosomes was from 1.29 to 2.41µm.

Although is assumed that B chromosomes derived from standard A chromosomes of either the same or related species (Huang *et al.,* 2016), some evidences suggest that B chromosomes were generated spontaneously as consequence of new genomic conditions after interspecific hybridization (Dhar *et al.,* 2019). In fact, it is estimate that 30–70 % of all flowering plant species have hybridization events in their phylogenetic histories (Neri *et al.,* 2018) and apparently that anthropogenic disturbances may be the major factor promoting hybridization, more over it can create a new ecological niche in which the hybrid can establish its populations (Li *et al.*, 2017).

Table 1. Comparative analysis of the karyotype of Trinidad, Penélope and Dulce varieties of *Tigridia pavonia*.

Variety	Chromosome number	Karyotypic formula	Total length of the Genome (LGT µm)	Asymmetry index (TF%)
Trinidad	2n = 28	28m	135.38[b]	46.16[a]
Penélope	2n = 28+3B	26m + 2sm + 3B	116.55[a]	44.41[a]
Dulce*	2n = 28+3B	26m + 2sm + 3B	124.94[a]	44.29[a]

LSD ($p \leq 0.05$).
*Data reported by Arroyo-Martínez *et al.* (2018)

In this sense it is noteworthy that the three varieties analyzed in the present study grow wild in the same geographic zone (2200 m.a.s.l.), usually in Pinus or Quercus forest, or riparian vegetation. Also these varieties share the same flowering period from June to August and they are considered colonizing. As it is known, hybridization may enhance the colonizing behavior of certain species (López-Caamal and Tovar-Sánchez 2014) allowing that parental species may coexist through formation of a stable hybrid zone (Hall 2016). These reports strengthen the idea that Penélope could be a natural hybrid between Trinidad and Dulce varieties, especially because Penélope showed the same number of B chromosomes reported for Dulce (Arroyo-Martínez *et al.,* 2018). Likewise, Penélope showed intermediate values of LGT in comparison with the other two varieties (Table 1) however, a higher similarity with Dulce ($p \leq 0.05$) was observed. This coincide with reported by Piña-Escutia *et al.* (2010a) who found a narrow genetic distance between Penélope and Dulce using morphological and molecular analysis, which shows the close relationship between these two varieties.

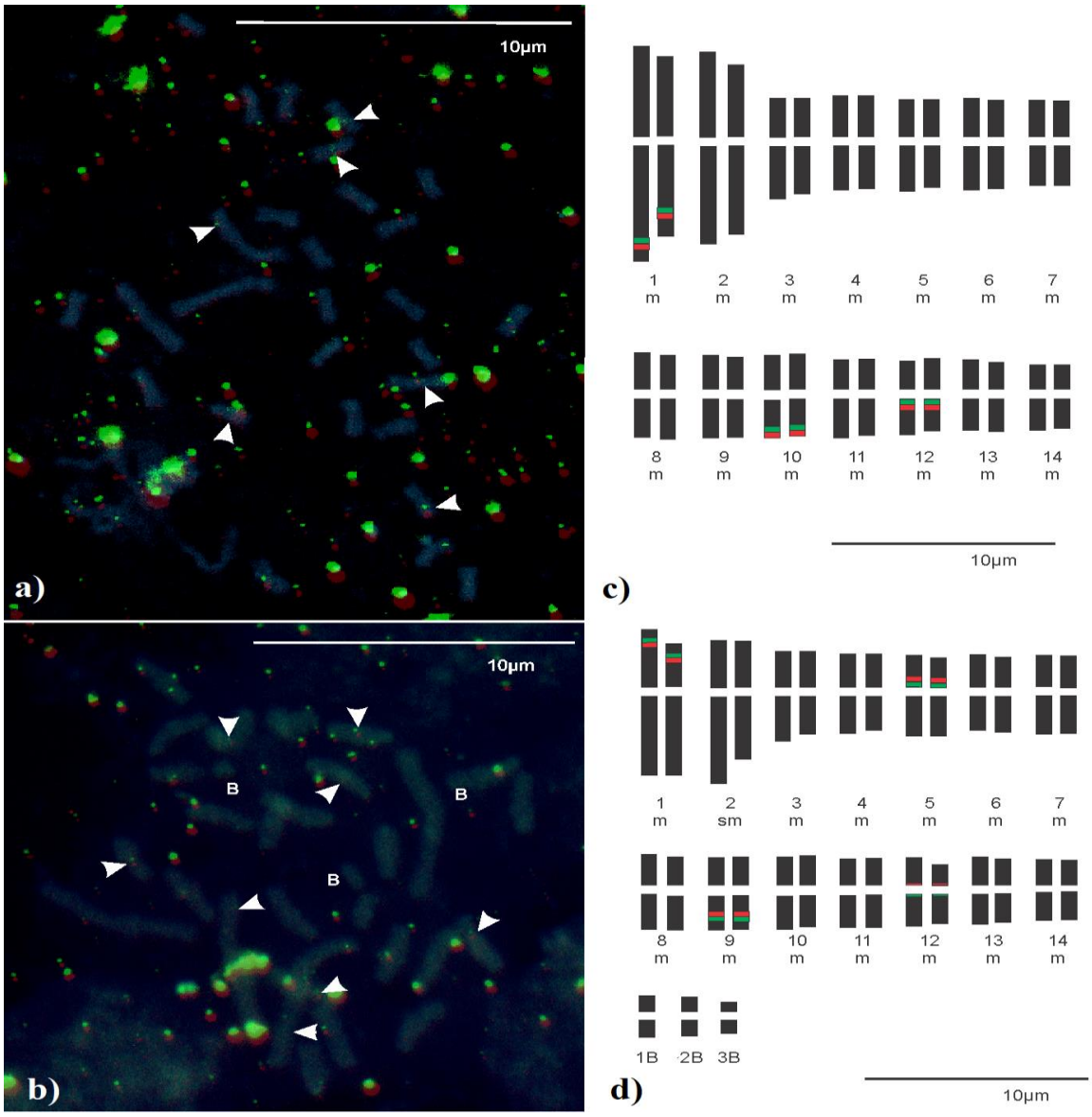

Figure 4. Fluorescent *In Situ* Hybridization of 5S (green) and 45S rDNA (red) genes in chromosomes of two *Tigridia pavonia* varieties: a) Trinidad, b) Penélope. Arrowheads indicate hybridization sites of both genes. c) Trinidad idiogram showing a karyotypic formula of 28m. d) Penélope idiogram showing a karyotypic formula of 26 m + 2 sm + 3 B. Red marks indicate 5S rDNA genes and green marks indicate 45S rDNA genes.

Barreto dos Reis *et al*. (2015) reported that the hybrid *Pennisetum purpureum* X *P. glaucum* showed DNA loss in relation to their parents, pointing out that it could be due to genomic alteration of the hybridization. In the present study similar results were found since chromosome length range of Penélope was different from those reported previously for Trinidad and Dulce (Arroyo-Martínez *et al*., 2018). Our results are also coincided with Riddle and Birchler (2003) who mentioned that union of two genomes in a single nucleus can alter the number and distribution of DNA sequences, which could cause genetic and epigenetic reorganizations, resulting in intergenomic conflicts and DNA loss.

Physical mapping of 5S and 45S rDNA genes

Labeling of repetitive sequences, such as ribosomal genes facilitate the identification of specific chromosomes of different species, allowing observe a specific distribution pattern that can be used as a karyotype marker for to enhances genomic differentiation among highly related species (Guidini *et al*., 2017; She *et al*., 2017; Zhao *et al*., 2017). In the present study similar results were found since Trinidad showed six hybridization sites for both genes (chromosomal pairs 1, 10 and 12) two of them were found in two of the large chromosomes and the rest in the small ones (Figure 4ac). Interestingly Penélope also showed two hybridization sites of both genes in two of the large chromosomes and the rest in the small ones (Figure 4bd), contrary to reported for Dulce where none hybridization site corresponded to large chromosomes (Arroyo-Martínez *et al.,* 2018), this suggest that Trinidad and Penélope could share similar genetic accommodation of their DNA sequences. Our results also coincide with reported by Piña-Escutia *et al.* (2010b) who observed a narrow genetic distance between Trinidad and Penélope when these were evaluated with RAPD markers.

López-Caamal and Tovar-Sánchez (2014) mention that chromosome number of hybrids is not a reliable tool when used in the absence of additional data, however it may provide robust hypothesis of hybridization when morphological or DNA fingerprinting techniques are used. Thus, considering that the three varieties coexist in the same geographic area, the intermediate values in flower size and percentage of fruit formation that Penélope presents in relation to Trinidad and Dulce (Vázquez-García *et al.*, 2001), the genetic similarity among these varietes (Piña-Escutia *et al*., 2010ab), and the cytogenetic results observed in the present study, it can be inferred that Trinidad and Dulce varieties could act as parents of the Penélope variety, although more specific analyzes like Genomic *In Situ* Hybridization or the use of molecular markers are required to confirm this.

CONCLUSIONS

In the present work it was possible to observe a high cytogenetic similarity among Penélope, Trinidad and Dulce suggesting that the latter may be the parents. Likewise, the karyotype of the Penélope variety is reported for the first time, with a chromosome number of 2n = 2x = 28 + 3B, a karyotypic formula of 26m + 2sm + 3B, showing eight hybridization sites of the 5S and 45S rDNA respectively. Trinidad presented a chromosome number of 2n = 2x = 28 with a karyotypic formula of 28m and showed six hybridization sites of both 5S and 45S rDNA genes.

Acknowledgements
We thank to Plant Biotechnology team of the Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco A.C. (CIATEJ) for aid in carrying out part of the research.

Funding. This investigation was financed for the Consejo Nacional de Ciencia y Tencnología (CONACYT) through the project 243266.

Conflict of interest. The authors confirm that this is an original work that is not considered for publication in any other journal, which does not present any conflict of interest that may inappropriately influence the work of persons or institutions.

Compliance with ethical standards. The research was conducted according to the established procedures of the Universidad Autónoma del Estado de México, under the authorization of the project CONACYT 243266.

Data availability. Data are available with the corresponding author Dr. José Luis Piña Escutia at: (jlpinae@uaemex.mx) upon reasonable request.

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