



**Citation:** Viviana Franco-Florez, Sara Alejandra Liberato Guío, Erika Sánchez-Betancourt, Francy Liliana García-Arias, Víctor Manuel Núñez Zarrantes (2021) Cytogenetic and cytological analysis of Colombian cape gooseberry genetic material for breeding purposes. *Caryologia* 74(3): 21-30. doi: 10.36253/caryologia-1081

**Received:** September 16, 2020

**Accepted:** July 20, 2021

**Published:** December 21, 2021

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**Data Availability Statement:** All relevant data are within the paper and its Supporting Information files.

**Competing Interests:** The Author(s) declare(s) no conflict of interest.

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## Cytogenetic and cytological analysis of Colombian cape gooseberry genetic material for breeding purposes

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**Abstract.** The cape gooseberry, *Physalis peruviana* L., is a crop that is transitioning from a semi-wild rural food source to becoming an international export commodity fruit deserving of greater attention from the scientific community, producers, policy makers, and opinion makers. Despite its importance, the crop has serious technological development challenges, mainly associated with the limited supply of genetically improved materials for producers and consumers. To bridge this gap, the present study determined the level of ploidy of 100 genotypes of cape gooseberry from a working collection by counting the number of chromosomes and chloroplasts, to include them in the breeding program. The number of chromosomes in dividing cells of root-tip meristems, as well as the number of chloroplasts per guard cell, from plants grown under *in vitro* and *ex vitro* conditions were determined. Haploid (with 24 chromosomes), doubled haploid, tetraploid (with 48 chromosomes), aneuploid (44 and 49 chromosomes), and mixoploid genotypes (with 36 to 86 chromosomes) were found. The number of chloroplasts per guard cell ranged from 4-8, 6-16, 7-16 and 9-21 for the haploid, aneuploid, doubled haploid-tetraploid, and mixoploid genotypes, respectively. The results showed evidence of a high cytogenetic diversity in the evaluated genotypes.

**Keywords:** chloroplast number, chromosome number, mixoploidy, *Physalis peruviana*, plant breeding.

### 1. INTRODUCTION

Colombia is the world's largest producer of cape gooseberry (*Physalis peruviana* L.), with a high-quality fruit desired for its aroma and flavor. During 2019, approximately 8,287 tons were mainly exported to the Netherlands, United Kingdom, United States, Canada and Belgium (Agronet, 2019; ANALDEX, 2019; PROCOLOMBIA, 2020). This makes cape gooseberry a crop with great competitive advantages for Colombia, which being a tropical country can guarantee yearlong production to supply the international market (Cotes et al., 2012). However, scientific, and technological progress is still lacking to

position it as a stable and competitive crop in Colombian agriculture. One way to address the agronomic and crop quality constraints is through the generation of genetically improved varieties for commercial production.

The genetic pre-improvement of cape gooseberry in Colombia – also known as *uchuva* in Colombia, *uvilla* in Ecuador, and *aguaymanto* in Peru – is a relatively new activity since currently there are just two genetic improvement programs contributing to the solution of agronomic problems of the crop. One of these programs, at AGROSAVIA, released the first two commercial varieties (Núñez et al., 2016b, 2016a) and the other, at the University of Nariño, is working toward the selection of genotypes for production under local conditions. However, the present commercial production process is mainly based on seeds of materials that producers select from their harvest. Therefore, improvements in the breeding process to enhance availability of superior production material is an important endeavor.

One essential prerequisite to breeding efforts is the elucidation of chromosome number in germplasm to be used as potential breeding parents. The ploidy level of the parents is a key factor that affects the efficiency of hybridization in the generation of new segregating populations, genetically stable in terms of chromosome number. In cape gooseberry, the ploidy variation has been supported at the cytogenetic level by several studies. Vilmorin and Simonet, (1928) determined a chromosomal complement of  $2n = 48$ ; Yamamoto and Sakai, (1932) described populations having  $2n = 24$  chromosomes; and Bracamonte et al., (1997) reported a chromosome complement of  $2n = 16$ , in which they named *P. peruviana* as “*capulí de la costa*” – a term sometimes used in commercialized cape gooseberry in Peru. Among the most recent cytogenetic studies of *P. peruviana* was that of Rodríguez and Bueno, (2006), who recognized variation in the number of chromosomes associated with different ecotypes, finding plants with a chromosomal complement of  $2n = 24$ ,  $2n = 32$ , and  $2n = 48$ . According to Lagos, (2006) the species presents three characteristic karyotypes:  $2n = 24$ , 36 and 48; three rare ones with 32, 38 and 40 chromosomes; and cases of mixoploidy. Bala and Gupta, (2011) stickiness of chromosomes, multivalents and univalents, and unoriented bivalents during metaphase-I, non-synchronization in the separation of some bivalents, laggards, chromatin bridges and cytomixis at various meiotic stages besides aberrant microsporogenesis. In spite of all these abnormalities, distribution of chromosomes at anaphases found to be normal. Microsporogenesis includes monads, dyads and tetrads with micronuclei besides normal tetrads, consequently reducing the pollen fertility (76%

reported that *P. peruviana* has intraspecific polyploid cytotypes that include diploids, tetraploids, octoploids and hexaploids. Recently, Trevisani et al., (2018) determined that Brazilian *P. peruviana* populations presented tetraploid cells  $2n = 4x = 48$ ; Carbajal (2018) found in three Peruvian ecotypes a ploidy level of  $2n = 4x = 48$  with chromosomal number variations in the same samples of analyzed cells in each of the studied ecotypes.

On the other hand, the Molecular Genetic Laboratory research group of AGROSAVIA has advanced in the analysis of the ploidy level of cape gooseberry by flow cytometry, chromosome and chloroplast counting (Franco, 2012; Liberato et al., 2014; García-Arias et al., 2018b). Franco, (2012) and García-Arias et al., (2018b) found chromosome numbers of  $2n = 4x = 48$  in the ecotypes Colombia and Kenya and chromosome variations induced by colchicine treatments; they additionally discovered that chromosome and chloroplast numbers were related to each other. Liberato et al., (2014) determined chromosome numbers of  $2n = 4x = 48$  and  $2n = 2x = 24$  that correlated with the nuclear DNA content estimated by flow cytometry. In addition, Berdugo et al., (2015) carried out crosses between some genotypes analyzed by Liberato *et al.*, (2014) and found distortion in the segregation, possibly related to differences in the chromosome size or chromosome number of these materials. The cytogenetic variability, found in the collection of the germplasm bank maintained at Agrosavia and in the working collections, highlights the importance of knowing the cytogenetic identity of each genotype before its inclusion in hybridization-based breeding programs. The ploidy knowledge of cape gooseberry genotypes is an essential factor in designing an appropriate breeding strategy (Trevisani et al., 2018) for the genetic improvement of the crop.

Therefore, the objective of the present study was to determine the ploidy level of one hundred genotypes of cape gooseberry using conventional chromosome and chloroplast counting techniques. These materials have been characterized by agronomic and quality attributes, including candidate genes associated with yield, size and fruit quality to develop superior genotypes (García-Arias et al., 2018a), and further exploring their ploidy level may inform the level of gene flow, and lead to the release of commercial improved varieties or hybrids.

## 2. MATERIALS AND METHODS

### 2.1 Plant materials

One hundred genotypes derived from the national germplasm bank, which is administered by the Cor-

poración Colombiana de Investigación Agropecuaria - AGROSAVIA (Table S1), were used for the study. The cape gooseberry population included wild genotypes, commercial genotypes from different producing areas of Colombia, ecotypes and germplasm obtained from *in vitro* anther culture. The plants maintained under *in vitro* culture conditions were sub-cultured in MS medium (Murashige and Skoog, 1962) modified with half of the nitrates ( $\text{NH}_4\text{NO}_3$  - 825 mg/l and 850 mg/l), under a temperature of  $25 \pm 2^\circ\text{C}$ , a light intensity of 2000 lux and a photoperiod of 16 light hours.

### 2.2 Determination of chromosome number

For chromosome counts, roots of *in vitro* plants were collected after 15 days of culture at 11:00 am, time of the day in which mitotic activity in the radical apices of *P. peruviana* is at its peak, as previously determined by Liberato *et al.*, 2014. Root tips of 2-3 cm in length were treated with 0.25% colchicine in a solution with 2% DMSO for three hours at room temperature. After this treatment, the root samples were fixed for 12 hours in Carnoy's solution (96% ethanol and glacial acetic acid in a 3:1 ratio). Subsequently, the roots were subjected to acid hydrolysis with 1N HCl for 25 minutes at room temperature. Finally, they were transferred to distilled water and kept for one hour at  $37^\circ\text{C}$ . The root tips staining was done on a slide with two drops of 2% propionic orcein for 15 minutes. Then, the tissue was crushed with a rubber bar. The cells were observed with the 40X and 100X lenses in an Olympus microscope to count the chromosome number in a sample of 25 cells per genotype.

### 2.3 Counting the number of chloroplasts

The chloroplast counts were performed on 25 guard cells using the methodology proposed by Orrillo and Bonierbale, (2009) in potato. Young leaves of each genotype were collected, and the epidermis was removed from the area close to the abaxial vasculature with sharp forceps. The sample was placed on a slide with two drops of iodine-potassium iodide (I-KI) solution in a 1:1 ratio in 70% alcohol. The preparation was observed under the microscope at 40X and 100X magnifications to determine the number of chloroplasts per stomatal guard cell.

### 2.4 Data analysis

To establish differential groups in relation to cytogenetic and cytological variability, a cluster analysis was performed using the Ward method (semi-partial  $R^2 =$

0.10), complemented with a Pearson correlation test ( $\alpha = 0.05$ ), using SAS® (Statistical Analysis System, Cary, North Carolina) version 9.3. Based on these analyzes, predictions were made on the possible results that could be found when carrying out intraspecific crosses between the genotypes studied.

## 3. RESULTS

### 3.1 Chromosome counting

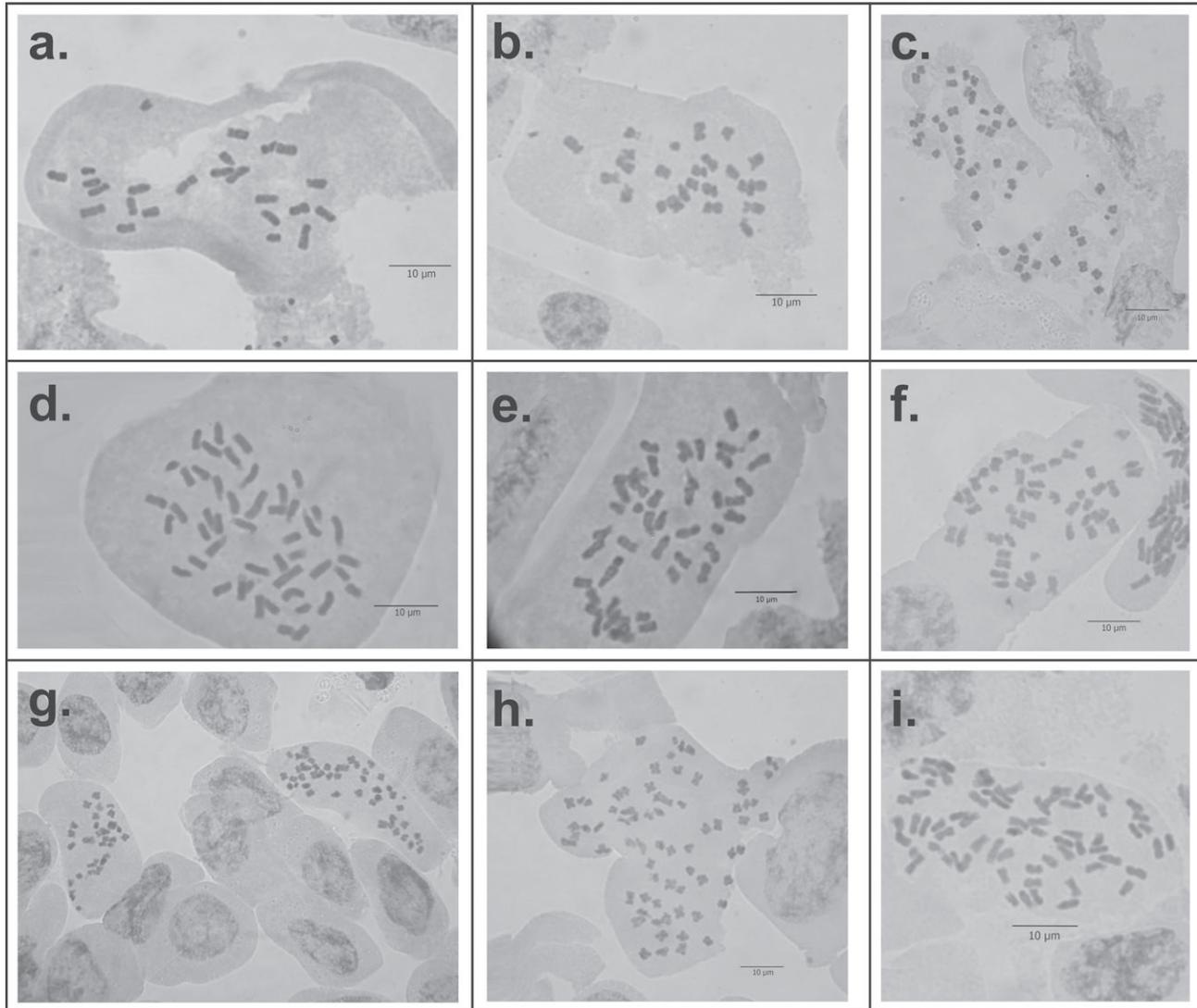
When counting the number of chromosomes of genotypes from the work collection, the basic chromosome number  $x = 12$  was predominant in the genotypes evaluated. We found that 85 of the 100 genotypes evaluated presented  $4x = 48$  chromosomes (Table S1 and Figure 1d, 1e -1f). Of these, 66 genotypes from commercial and wild populations were tetraploid, and 19 doubled haploid genotypes derived from anther culture. Seven haploid genotypes from anther culture showed  $n = 2x = 24$  chromosomes (Table S1 and Figure 1a-1b). Two aneuploids were observed: the genotype 09U012-5 with 44 chromosomes and the genotype 09U261-2 with 49 chromosomes (Table S1 and Figure 1c). Mixoploidy was also present in six genotypes (Table S1 and Figure 1g-1i) related to five plants from anther culture and the genotype 09U136-3 from a working collection. The mixoploid genotypes presented different chromosomal complements in the same plant, with counts ranging from 36 to 86 chromosomes (36, 38, 40, 44, 48, 49, 52, 54, 57, 58, 60-74, 76-78, 80, 82, and 86 chromosomes). These results indicate that the population of one hundred cape gooseberry genotypes studied has a high cytogenetic diversity represented by tetraploids, aneuploids, mixoploids, haploids and doubled haploids.

### 3.2 Chloroplast counts

The number of chloroplasts per guard cell of haploid genotypes derived from anther culture ranged from 4 to 8, the aneuploid genotypes presented between 6 to 16 chloroplasts, while tetraploids and doubled haploid genotypes presented between 7 to 16 chloroplasts. Additionally, the mixoploid genotypes ranged from 9 to 21 chloroplasts per guard cell. Table S1 and Figure 2 show the relationship of the chromosome and chloroplast counts of the analyzed genotypes.

### 3.3 Cluster analysis

According to the cluster analysis (Figure 3 and Table 1) the studied genotypes formed four groups. The first



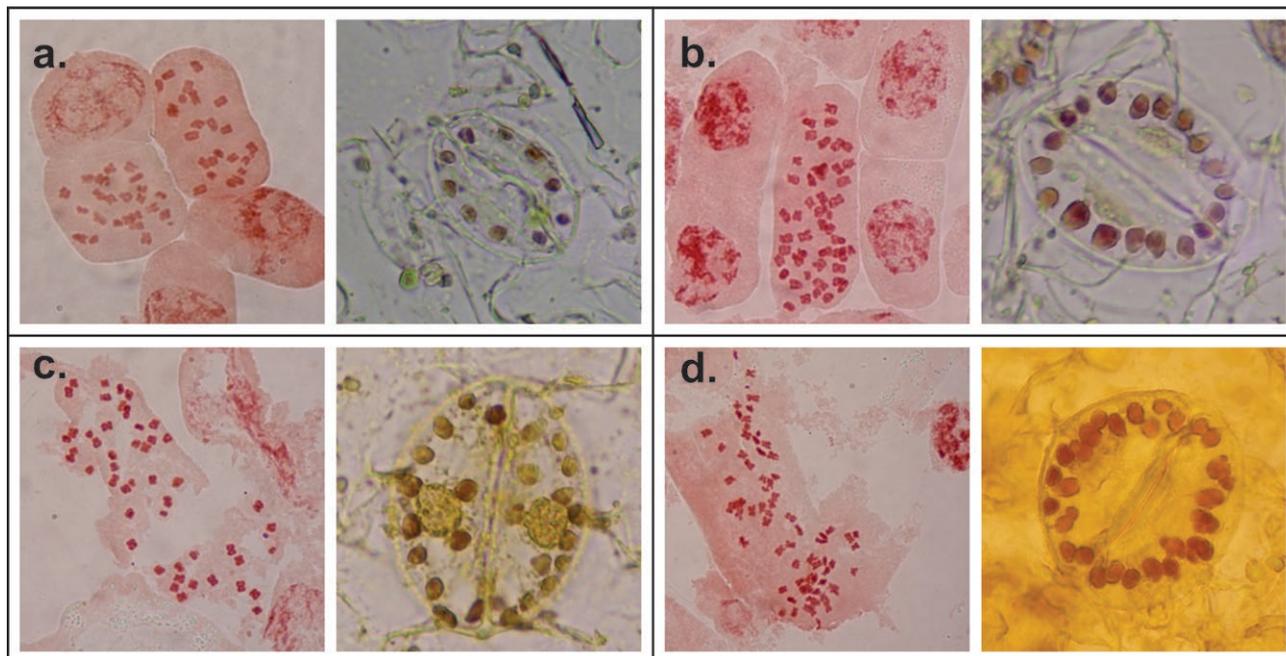
**Figure 1.** Karyotypes representative of *P. peruviana* genotypes. a. and b.  $n = 2x = 24$  genotypes 12U398 and 09U292-7. c. 49 chromosomes genotypes 09U261-2. d., e., and f.  $2n = 4x = 48$  genotypes 09U134-3, 09U048-1 y 09U120-3. g., h. and i. mixoploids 38, 48 y 71 chromosomes, genotypes 09U136-3, 09U296-2 and 14U422. Scale of 10µm. All the photos were taken at 100X except for image 1h, which was taken at 40X.

group was made up of seven haploid genotypes with an average of 5.69 chloroplasts. The second cluster grouped 35 genotypes corresponding to doubled haploids and tetraploids that presented a chloroplasts average of 11.04. The third group consisted of six mixoploid genotypes mostly derived from anther culture with a chloroplast average of 13.63. The fourth group was integrated by 52 genotypes that included doubled haploids, tetraploids and aneuploids with a chloroplast average of 9.82. Pearson correlation coefficient between the number of chromosomes and number of chloroplasts per guard cell was of  $r = 0.89$  ( $p < 0.0001$ ).

## 4. DISCUSSION

### 4.1 Diversity of chromosome counts

A population of one hundred genotypes of cape gooseberry from commercial, wild, working collection and anther derived plants were analyzed in this study. A wide range of cytogenetic variations was observed from the four sources of genotype samples. Specifically, the counts of 48 chromosomes from tetraploid genotypes found in this study coincide with that reported by Vilmorin and Simonet, (1928); Menzel, (1951); Gupta and



**Figure 2.** Number of chromosomes and chloroplasts per guard cell from some representative genotypes of *P. peruviana*. A. Somatic metaphase cell with 24 chromosomes and stomata with five chloroplasts. B. Cell with 48 chromosomes and stomata with nine and ten chloroplasts in the guard cells. C. Cell with 49 chromosomes and stomata with 12 chloroplasts in the left guard cell. D. Cell with 69 chromosomes and stomata with 17 chloroplasts in the upper guard cell.

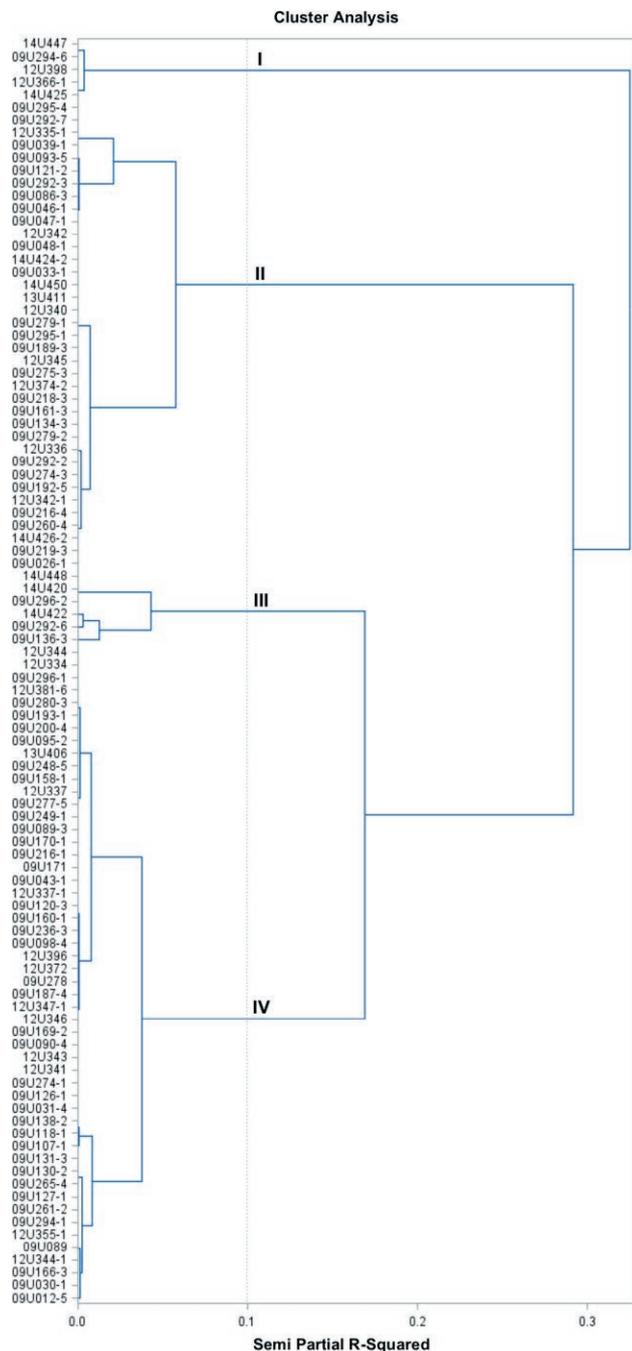
Roy, (1985); Moriconi et al., (1990), and Ganapathi et al., (1991). The results also agree with recently published studies that mention the 48-chromosome number as the most common event in *P. peruviana* L. genotypes from Brazil (Trevisani et al., 2018), Peru (Carbajal, 2018). The chromosome number  $2n = 4x = 48$  was also mentioned in the work of Lagos, (2006), who reported for *P. peruviana* chromosome numbers of 24, 36 and 48 as the three characteristic karyotypic constitutions for the species.

The results of this study also agrees with the 48 chromosome number of the Kenyan ecotype reported by Rodríguez and Bueno (2006) and with the results of Liberato et al., (2014) who determined  $2n = 4x = 48$  in several cultivated genetic material from Colombia. Seems that the 48 chromosome number is predominant in *P. peruviana* L. as shown by Trevisani et al., (2018) who reported the chromosome number  $2n = 4x = 48$  of four *P. peruviana* L. populations from Brazil, Colombia and Peru, classifying them as polyploid with tetraploid cells, which agrees with the results of this study. Among the genotypes analyzed there were several plants derived from anther culture with the genetic load of 48 chromosomes like their parents and defined as doubled haploid lines  $2(n) = 4x = 48$ . Reduction of chromosome number and double haploidization are events that occur through the process of androgenesis. This, may be due to spontaneous or induced chromosome

duplication of the microspore under *in vitro* culture conditions, as reviewed by Germanà, (2011).

Several studies have shown chromosome number related to diploidy level in several *P. peruviana* L. populations from different countries. Rodríguez and Bueno (2006), Lagos (2006) and Liberato et al., (2014) determined chromosomal constitutions of  $2n = 2x = 24$  in wild genetic materials from Colombia. While Azeez et al., (2019) and Azeez and Faluyi (2019) in two different studies found that *P. peruviana* L. has  $2n = 2x = 24$  chromosome constitution as compared to three different Nigerian *Physalis* species. In this study several genotypes derived from anther culture showed a reduction by half in the chromosomal load to the gametic number of 24, the same as the gametic chromosome number of the species. These anther culture derived genotypes are considered as haploid lines with  $n = 2x = 24$  ploidy. These results were also observed by Escobar et al., (2009) in a study of anther culture with different cultivars of Mexican husk tomato (*P. ixocarpa* Brot.).

In the present study the results also show other chromosome numbers in several genotypes related to mixoploidy and aneuploidy, quite different from those tetraploids, diploids, doubled haploids and haploids genotypes analyzed. The mixoploid and aneuploid nuclei observed in several sample plants were not only observed in gen-



**Figure 3.** Dendrogram of grouping by number of chloroplasts in stomatal cells and chromosome number of 100 genotypes of *P. peruviana*.

otypes from anther culture derived plants, but also in plants from working collections derived from germplasm bank main collection. Our results although do not show the same chromosome number for mixoploidy and aneuploidy, agree with the several published reports in *P.*

*peruviana* L. Rodríguez and Bueno (2006) found that Colombia ecotype had  $2n = 3n = 32$  chromosome number and Lagos, (2006) reported chromosome numbers of 36. The results suggests that in cape gooseberry not all the cells analyzed from the same plant sample have the equal chromosome number, as already shown by Carbajal (2018) who reported that 30%, 40% and 50% of the analyzed cells showed aneuploidy, with chromosome number range from 44 to 80 quite different from the commonly observed chromosome number of  $2n = 4x = 48$ . Mixoploidy is a very promising source to produce haploid plants in short time from somatic tissue. Haploid plants once their genome is duplicated double haploid plants can be generated and used in genetic studies and crop improvement.

In plants derived from anther culture or isolated microspore that arise through the process of androgenesis, besides expecting haploids and doubled haploids genotypes, for the case of *P. peruviana*, aneuploidy and mixoploidy are also generated, as was observed in our results. Zagorska et al., (2004), mentioned that the variation in the chromosome number in gametes or gametophytic tissue plays an important role for gametoclonal variation during *in vitro* androgenesis and can result in haploid, doubled haploid, tetraploid, aneuploid and mixoploid plants. The variation in chromosomal contents of the plants derived from anther culture might be related to aberrant cell division in the immature pollen grains due to the stress generated by the *in vitro* culture conditions. This phenomenon was observed by Sánchez, (2014) and García-Arias et al., (2018b) in cape gooseberry anther culture-derived plants and other species such as *Solanum lycopersicum* (Arcobelli et al., 2014) and *Humulus lupulus* (Koutoulis et al., 2005). However, plants that come from natural populations as is the case of the genotype 09U136-3 of this study was mixoploid despite being from natural populations; suggesting that mixoploidy occurs naturally in cape gooseberry because of the continuous evolving process of the specie that is still under domestication. To this respect, Trevisani et al., (2018) states that *Physalis peruviana* possibly has not fixed its chromosomal structure yet.

The aneuploid genotype 09U012-5 with 44 chromosomes analyzed in this study agrees in chromosome number with the ecotype Peru reported by Franco, (2012), while the genotype 09U261-2 with 49 chromosomes corresponds to a new report for *P. peruviana*. These chromosomal loads do not have a direct relationship with the basic number  $x = 12$  reported for the genus *Physalis* (Menzel, 1951; Gupta and Roy, 1985; Ganapathi et al., 1991; Lagos, 2006, Trevisani et al., 2018 and Carbajal, 2018). Therefore, these genotypes could arise from

**Table 1.** Cluster analysis. Conformation of groups by chromosome number.

Group	Chromosomal number						Total
	Haploid n = 2x =24	Doubled haploid 2(n) = 4x =48	Tetraploid 2n = 4x =48	Aneuploid		Mixoploid 36-86	
				44	49		
1	7	0	0	0	0	0	7
2	0	8	27	0	0	0	35
3	0	0	0	0	0	6	6
4	0	11	39	1	1	0	52
Total	7	19	66	1	1	6	100
Chloroplast average	5,69	10,40	10,27	9,34		13,63	
Chloroplast range	4-8	7-16	7-16	6-16		9-21	

\* Number of genotypes that present determined chromosomal number.

the restructuring chromosomal set that involves the gain or loss of a chromosome (aneuploidy), structural rearrangement of chromosomes resulting in the increase or decrease in chromosome number, or hybridization between polyploids with different chromosome numbers (Poggio and Naranjo, 2004).

#### 4.2 Chloroplast counting as a proxy for ploidy level determination

The number of chloroplasts found in the haploid genotypes from this investigation agrees with results reported by García-Arias et al., (2018b) in *P. peruviana* haploid plants obtained from anther culture as well. In that report, the authors specified that those plants had 4-7 chloroplasts per guard cell. In an independent study, Franco, (2012) working with three ecotypes reported ranges of 7-12 chloroplasts per guard cell for Peru, and 8 to 13 chloroplasts for the Colombia and Kenya, the same as the results shown in the present study.

Additionally, the Pearson correlation coefficient presented in this study is higher than the one reported by García-Arias et al., (2018b), who showed a correlation of  $r = 0.61$ , probably due to differences in the plant material analyzed and the sample size. In both research works a relationship between chloroplast number and chromosome number, that suggest that the chloroplast number is influenced by de chromosomal content of a genotype in a proportional manner, as shown by Rodriguez and Bueno, (2006). Therefore, the chromosomal variation, can affect the phenotypic characteristics of a genotype, justifying a direct proportional relationship between the level of ploidy and the size of different organs of the plant. Recently García-Arias et al., (2018b) reported that the increase of the number of chromosomes of haploid

sterile cape gooseberry plants, is directly associated with the recovery of pollen fertility, variation in morphology of the leaf, flower bud, flower and normal fruit set.

The results of the cluster analysis showed four groups based on chromosome and chloroplast number, differentiating the haploid, doubled haploid, tetraploid and mixoploid genotypes (Table 1). A mixture of doubled haploid and tetraploid genotypes in the cluster two, formed two subgroups due to the amplitude of the range in the number of chloroplasts. The third group was composed of mixoploid genotypes and the fourth group was composed of tetraploid and aneuploid genotypes. These results suggest that ploidies can be estimated primarily by counting the number of chloroplasts in guard cells, but not in an exact way, since the aneuploid, doubled haploid and tetraploid genotypes formed a single group due to overlapping chloroplast count totals.

#### 4.3 Crossability strategies

The ploidy level is very important for plant breeding and crop improvement strategies (Udall and Wendel, 2006; Ochatt, 2011). This highly relates with natural divergence among subpopulations and the scale of local adaptation, as well as to the capability of gene flow and crossability in plants for successful interspecific and intraspecific hybridization. The observed chromosomal variations of 44, 48 and 49 can occur without noticeable changes in the phenotype (Poggio and Naranjo, 2004), while the cases of mixoploidy generate evident phenotypic changes. In *P. peruviana*, Franco, (2012) and García-Arias et al., (2018b) found an amorphous development in flowers and fruits, changes in the floral and vegetative structure, and an increase in the size of the fruit associated with mixoploidy events.

In crosses between genotypes with chromosomal dissimilarities, in terms of shape, size and number, there may be no fertilization or abortions due to irregularity in meiosis, a situation reported by Menzel, (1951) in other *Physalis* species. Serrato-Cruz et al., (2000) and Laguado, (2007) unexpected ploidy levels, genomic instability, or odd chromosome numbers in hybrid progenies between parents with different ploidy level, which could lead to events of unreduced gametes, apomixis, and chimeras, leading to infertility and low levels of productivity. The genotypes which chromosomal complement have broad similarity can be successfully crossed since a normal meiotic division would occur, as Menzel, (1951), Ortiz et al., (1998) and Serrato-Cruz et al., (2000) pointed out. Consequently, we could carry out intraspecific crosses among genotypes with the same chromosomal numbers, expecting to obtain viable and stable progeny. A precedent example of the success of crossing between genotypes with the same ploidy is the work of Berdugo et al., (2015). In their study the authors made intraspecific crosses among accessions of *P. peruviana* with the same chromosome number, finding 100% viability in the progeny. In general, chloroplast counting could be useful as a quick method to assess the ploidy when the number of genotypes to be analyzed is large.

## 5. CONCLUSIONS

In this study, there was a close relationship between chromosome and chloroplasts number per guard cell, which was confirmed through the cluster analysis. It is clear, that the chloroplast count is an approximate and quick methodology to be used as a preliminary way for determination of the ploidy level, when dealing with many genotypes. As far we know, these are the first study that analyzed one hundred genotypes of *P. peruviana* L. from different sources for breeding purposes. In summary, this work contributes to the cytogenetic and cytological knowledge of cape gooseberry and is a useful tool in the selection of crossing parents in breeding programs.

## AUTHOR CONTRIBUTIONS

VMNZ and EPS-B conceived the study. VF-F, SAL-G realized the experimental work. VF-F, SAL-G and FLG-A performed statistical analysis and prepared the first manuscript, VMNZ refined the final version. All authors read and approved the final version of the manuscript.

## ACKNOWLEDGMENTS

We thank the Corporación Colombiana de Investigación Agropecuaria - Agrosavia and MinCiencias for their financial support.

## FUNDING

This work was supported by the Corporación Colombiana de Investigación Agropecuaria - Agrosavia and MinCiencias in the project “Nuevos cultivares de uchuva con características genéticas, fisiológicas y fitosanitarias con énfasis en resistencia a *Fusarium oxysporum* f. sp. *physali* y calidad de fruto”.

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