Anatomical tool in the identification of ploids in maize seedlings and potential use in initial stage of double haploides obtainment process

Anatomical tool in the identificantion of ploids and potential use of double haploides in maize

Raquel M. O. Pires[1]*, Genaina A. Souza[2], Danielle R. Vilela[1], Heloisa O. Santos[1], Renato C. C.Vasconcellos[1], Édila V. R. Von Pinho[1]

1 Department of Agriculture, Universidade Federal de Lavras, Lavras, Minas Gerais, Brazil,

2 Department of Vegetal Biology. Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil.

*Corresponding author

E-mail: raquelmopires@ufla.br

Abstract

Studies that optimize the haploid technique in the removal of maize lines are necessary. Between the stages that mostly requires attention and it is directly related to the success of the technology is the correctly separation of induced haploids and diploids. Morphological markers are commonly used but have strong influence of the environment, and laboratory methods have been developed and may be more efficient. Thus, the objective was to study the use of the anatomical analysis tool, through the analysis of young maize leaf for use as the indirect markers in the identification of ploidys. The hybrids were crossed with the KEMS haploid inducer. The seeds crossed, were selected according to the R-navajo marker and submitted to two different protocols of chromosome duplication. Plants that survived to the duplication protocols were acclimated in greenhouse and then transferred to the field. After the self-polinization of the DH0 plants, the DH1 seeds were taken to the field, divided into treatments according to the parentals and duplication protocols. At the vegetative stage V4 of the plants, leaf tissue samples were collected to the evaluation of the amount of DNA and identification of ploidys and anatomical analysis. The nuclear DNA review of each sample was performed for the comparison in histograms of the position of G1 peak to the G1 peak of the internal or external reference standard. A high accuracy came to validate an anatomical tool, through the variables studied in this work, as a marker in the differentiation of ploidis in maize plants, and it can be used in selection programs. The anatomy made in some letters is a non-destructible technique and, together with a flow cytometry technique, can be used as an indirect method in haploid cutting programs at the initial stage of the identification of seedlings.

Introduction

The success of a breeding program that aims at the production of maize commercial hybrids lies in the fact of obtaining elite lines. Among all the steps, this is considered to be the most time-consuming and costly, and the technology of double haploid emerges as a way of reducing time in obtaining these lines [1].

The rapid production of homozygotic lines allows a better exploitation of genetic variability and increases the efficiency of selection. Homozygous plants will have the maximum additive variance, the effects of dominance and epistasis, and the advantages in the selection of quantitative, superior characteristics [2]. In addition, the decrease of the costs with labor, use of smaller experimental area and anticipation of profits in commercial programs for maze breeding have made this technique a great success.

The production of double- haploid lines involves four main steps: in vivo induction of haploidy, identification of possible haploids, chromosome doubling and the self-fertilization of lines obtained for increment of seeds [3]. However, the success of this methodology is still dependent on the use of inductors with high capacity of induction, a precise system of identification and differentiation of haploid and diploid seeds, as well as efficient and reproducible protocols of chromosome doubling [4].

The doubling of the chromosome number spontaneously or induced by the application of mitotic agents e.g., colchicine, retrieves the diploid condition and restores fertility [5]. The action mechanism of colchicine involves the irreversible connection to tubulin dimers, causing a conformational change and preventing the polymerization of mitotic spindle, and as a result, the newly duplicated chromosomes are not separate and the core reorganizes with the number of duplicated chromosomes [6]. However, not all cells of the treated tissue polyploidize, which can lead to the formation of chimeras, i.e., tissues or plants with duplicate sectors and others unduplicated ones [7] called mixoploids. Truly duplicated lines resulting from this process are called duplicate or double haploids (DHs).

There are several methods for certification of polyploidization, being flow cytometry the most used one [8]. Flow cytometry is a reliable and fast method, because it allows the analysis of a large number of cells and of different tissues [9]. In experiments with double haploids, flow cytometry allows vigorous seedlings and detected as diploids in the histograms are discarded before the step of field, reducing time and space. In addition, flow cytometry allows the analysis of the efficiency of the protocol of chromosome doubling, since it is not possible to confirm if the response of seedlings to duplication was positive [10–12].

Another tool that is being studied as a marker in the differentiation of ploidies in plants is the leaf anatomy, being the leaf considered the component with greater ability to adapt to environmental conditions. Highly flexible, leaf anatomy is influenced by environmental factors, such as, irradiation (leaves of sunlight/shade, [13], nutrients [14], drought [15] and ozone [16,17]. Changes in the leaves characteristics, such as those related to the thickness of the leaf blade, parenchymas, epidermis and number of stomata for example, and that are highly associated with the photosynthetic potential of plants, are used in studies of genetic selection by the use of morphological and anatomical markers. Additionally, they are highly heritable characteristics, i.e., can be passed to their offspring [18].

The cytoanatomic characterization is a methodology that allows the identification of haploid and supposed polyploidy in plants subjected to chromosome doubling. The study of measurement and comparison of stomata, based on the principle that the length of the same normally increases with the number of chromosomes, is the most commonly cited in the literature [19].

The number of stomata in association with other leaf anatomical characteristics, has already been mapped to different levels of ploidy in studies with coffee plants [20]. For this species, the greater the number of stomata the higher the ploidy. In the case of *Coffea canephora*, a reduction in the stomatal density is higher in the tetraploid level for some cultivars [20]. [21], observed in Citrus that the size and density of stomata varied according to ploidy level, where the triploids showed a higher number of stomata when compared to

105 diploid plants. A similar result was observed by [22], who stated to be possible the use of
106 anatomical markers for purposes of selection of citrus with different levels of ploidy.
107 Ploidy is well studied from the point of view of genetics and genomic perspective, but the
108 morphological and anatomical aspects related to these differences in the amount of DNA,
109 remain poorly studied in maze plants. Analyzing the anatomical characteristics of young
110 leaves of maize, capable of discriminating the different ploidies and extrapolate these results
111 in diploid and haploid discrimination on the optimization of the process of obtaining double
112 haploid is of extreme importance.
113 Thus, the objective of this work was to study the use of anatomical tool, through the
114 analysis of the characteristics of young leaves of maize for use as indirect markers in the
115 identification of ploidies, and through future studies, to extrapolate the use of this marker in
116 the identification of haploids in the initial stage of the process of obtaining double haploids.
117

118 # Material and methods

119 The seeds used in this work were obtained from an experiment previously developed by
120 [23] through the cross between four simple hybrids (DKB393, GNS 3225, GNS 3264, GNS
121 3032) with the haploid inducer KEMS, used as male parental. Seeds from these crosses
122 were separated by staining of the embryo and endosperm and selected as possible haploids
123 according with the marker R-navajo [24].
124 The authors submitted the haploid seeds to two chromosome duplication protocols, and
125 the plants that survived the field, called DH0, that produced pollen and had stigma style in
126 synchronism, were self-fertilized, resulting in DH1 generation.
127 Thus, in this present work, in order to evaluate the maintenance of DH in future
128 generations, the DH1 ears were harvested and the seeds threshed and dried at room
129 temperature up to 12% moisture level at the point of physiological maturity. The seeds were
130 then mixed and divided into treatments as shown in Table 1. These seeds were then stored
131 in a cold chamber at 10 ° C until the following experiments were carried out.

Table 1. Treatments taken for the field in the harvest season 2014/2015, established in accordance with the parental and the protocols of chromosome doubling.

Materials Identification		
Treatments	**Hybrids**	**Protocols**
1	DKB393	1
2	DKB393	2
3	GNS3225	1
4	GNS3225	2
5	GNS3264	1
6	GNS3264	2
7	GNS3032	1
8	GNS3032	2

In the harvest season 2014/2015, the total number of seeds DH1 for each treatment, was taken to the field and the experimental design was a randomized complete blocks with eight treatments with four replications. Each block was composed by lines of 10 meters of length, with spacing of 80 cm between rows and among plants of 25 cm, and sowing of a seed per hole. The collection of leaf material occurred in young stage of the plant maize, with 4 replicates collected at random within each treatment.

In the case of leaf anatomy, 5 replicates were performed for each repetition in the laboratory, five blades of 10 sections each, were made, and the top five fields were photographed and subsequently measured. The following were measured: thickness of the leaf blade (ELF), thickness of the parenchyma (PAR), thickness of the upper epidermis (EES) and lower epidermis (EEI), polar diameter of the stomata in the upper face (DPS) and lower (DPI), equatorial diameter of the stomata of superior face (DES) and lower face (DEI) and stomatal density on the upper surface (DS) and lower (DI). For that, in the vegetative

148 stage V4 which corresponds to the development of four leaves, leaf tissue samples were
149 collected.

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151 leaves, leaf tissue samples were collected.

152 For both evaluations, the medial portion of the young leaves fully developed, was cut
153 into segments of approximately 3 cm in length and wrapped in aluminum paper duly
154 identified per plant. Aluminum papers remained in a polystyrene box containing recyclable
155 crushed ice, until the time of transportation to the laboratory of tissue culture of plants of the
156 Department of Agriculture of UFLA.

157 At the opening of each envelope of aluminum, part of the samples of leaf tissue was
158 used for the quantification of DNA and part to the anatomical analysis. Therefore, young leaf
159 tissue samples of maize were crushed under ice, in Petri plates containing 1 mL of cold
160 buffer LB01, to obtain nuclear suspension [25], which was added 2,5µL RNase and stained
161 with 25 µL of propidium iodide (1 mg mL-1). The species *Vicia faba* (quantity of DNA of 26.9
162 pg/2C) was used as an external standard of reference and for each sample at least 10
163 thousand cores were analyzed. Each suspension was analyzed in flow cytometry
164 FacsCalibur (Becton Dickinson). The histograms obtained were evaluated by the WinMDI
165 software 2.8 (2009) for the evaluation of the peaks of DNA. The estimate of the nuclear DNA
166 content (pg) of each sample was performed by comparing the position of the peak G1 with
167 the peak G1 of the internal standard or external reference.

168 For this comparison the following expression was used:

169

170 $$Q = \left(\frac{E}{S}\right) x\ R \qquad (1)$$

171

172 Where:

173 Q is the quantity of DNA of the evaluated sample (pg/2C).

174 E is the position of the G1 peak of the sample.

S is the position of the peak G1 reference standard and

R is the quantity of DNA of the standard sample (26.9 pg/2C).

By the quantity of DNA it was possible to make inferences about the ploidy level of the evaluated genotypes.

As mentioned, the other part of the samples of leaf tissue was immediately fixed in FAA_{50}, (formaldehyde: acetic acid: ethanol, 5:5:90) for 48 h. Then, the samples were removed from the fixative solution, rinsed and stored in 70% ethanol solution [26]. At this moment the samples were transported to the Laboratory of Anatomy and morphogenesis of Plant Biology Department of the Federal University of Viçosa, where permanent slides were made using portions of the leaf that were dehydrated in ethyl series included in historresin (methacrylate), according to the manufacturer's recommendations. Transverse sections of leaf were sectioned in automatic advance rotary microtome (model RM2155, Leica Microsystems Inc., Deerfield, USA) with 5 µm thick, arranged on histological slides and stained with toluidine blue [27], to the limbal micro morphometry.

For the stomata evaluations, fragments of the central part of the leaf blade were sectioned from material stored in alcohol 70%. The samples were clarified by means of the technique of Diaphanization, described by [28] and modified for the species, having been clarified in methanol for 48 hours and then in lactic acid for 6 hours in water bath for 98 ºC and assembled into lactic acid. After the staining procedure, the samples were immersed in ethanol 80, 70 and 50%, and later were rinsed in distilled water. The histological slides with leaf fragments were mounted on glycerol-jelly Images of the slides of cross cuts and diaphanization were obtained under a light microscope (model AX-70 TRF, Olympus Optical, Tokyo, Japan) coupled to a digital camera (model Zeiss AxioCam HRc, Göttinger, Germany) and a microcomputer with the program to capture images Axion Vision, having been digitized and stored in a microcomputer. For the analysis, 10 distinct fields of each sample were measured by means of Image-Pro® Plus software (version 4.1, Media Cybernetics, Inc., Silver Spring, USA).

The analyzes were performed for all characters with the estimation of variance components and the prediction of random effects using the approach of mixed models, the method of restricted maximum likelihood/best linear unbiased prediction (REML/BLUP) [29]. For this, the following statistical model was used:

$$y = Xr + Za + Wp + e \quad (2)$$

Where:

y: vector of data;

R: vector of the effects of repetition (assumed as fixed) added to the overall average;

A: vector of genotypic effects among treatments (random), being a ~ NMV (0, Iσ_g^2). O σ_g^2 is the variance associated with genotypic among treatments;

p: vector of genotypic effects among treatments (random), being p ~ NMV (0, Iσ_p^2). O σ_p^2 is the variance associated with the parcels effects;

e: vector of random errors, and ~ NMV (0, Iσ_e^2). O σ_e^2 is the variance associated with the residual effects;

X, Z and W: incidence matrices for r, and p, respectively.

The heritability in the average of treatments (h_m^2) and accuracy were estimated, and the significance of the random effects as the genotypic variance among treatments (σ_g^2) were tested by the *Likelihood Ratio Tests* (LRT) where the analyses of deviance were obtained for each character evaluated [30].

These statistical analyzes were performed using the software SELEGEN-REML/BLUP [29].

Results

For all the evaluated characteristics the accuracy was classified as very high [31], with values above 87.0%. On average, the heritability (h_m^2) of the characteristics was also high with values above 84.0%, what indicates good reliability of data (Table 2). The heritabilities of treatments ranged from 76.0% (EES) to 98.0% (DEI) among the evaluated characteristics, which demonstrates that in the anatomic evaluation in this experiment, the largest part of the variation observed is due to genetic causes to the detriment of environmental variations [32].

The genotypic variance was highly significant by the LRT test and likelihood ratio for the characters related to the thickness of the leaf blade (ELF), stomatal density of both the upper surface (DS), as well as the lower surface (DI), thickness of the parenchyma (PAR), thickness of the upper epidermis (EES) and lower epidermis (EEI), polar and equatorial diameter of upper epidermis (DES and DPS) and lower (DEI and DPI), according to LRT ($P < 0.01$), characterizing these characteristics as good candidates to be inserted in a breeding program as markers. It was possible to realize that the characteristics related to the thickness of the lamina and those related to the stomata were the ones that showed higher heritability, followed by the thickness of the parenchyma. The thickness of the epidermis, even with high heritability, were lower (Table 2).

Table 2. Summary of ANADEV and estimates of genetic parameters in the evaluation of maze hybrids for traits. Thickness of the leaf blade (ELF), thickness of the upper epidermis (ESS), thickness of the parenchyma (PAR), thickness of the lower epidermis (EEI), equatorial diameter of the stomata in the upper face (DES), polar diameter of the stomata in the upper face (DPS), Equatorial surface of the stomata in the lower face (DEI), polar diameter of the stomata of the lower phase (DPI) and stomata densities of upper surfaces (DS) and lower (DI) of the leaf.

Parameters	ELF	EES	PAR	EEI	DES	DPS	DEI	DPI	DS	DI
$VG_{between}$ ($\hat{\sigma}_g^2$)	0.000235**	0.000011**	0.000092**	0.000007**	0.000013**	0.00001**	0.000024**	0.000011**	44.539206**	286.20**
LRT among treatments	82.36	14.84	60.19	28.25	134.79	125.70	200.61	189.59	20.36	67.65
VG_{within} ($\hat{\sigma}_p^2$)	0.000002 ns	0.000001 ns	0.000001 ns	0.000000 ns	0.000000 ns	0.000000 ns	0.000000 ns	0.000000 ns	0.8338 ns	1.2856 ns
LRT within treatment	0.00	0.01	0.00	0.00	0.01	0.01	0.07	0.00	0.01	0.00
h^2_{mg} (%)	93.2**	76.2**	91.0**	84.2**	96.8**	96.2**	98.1**	97.3**	84.8**	94.9**
AC_{gen} (%)	96.6	87.3	95.4	91.8	98.4	98.1	99.0	98.7	92.1	97.4

** Significant and ns Non significant by the likelihood ratio test (LTR), with 1% of probability.

The anatomical differences observed were obtained through anatomical cuts and diaphanization of samples. It was possible to observe a difference in leaf blade as the thickness (Fig 1) and quantity and size of the stomata (Fig 2).

Fig 1. Cross-sectional cuts of maze leaves collected after a process of chromosome doubling with use of colchicine. Haploid material (A), diploid (B), Double haploid (C), triploid (D) and tetraploid (E). ELF= thickness of leaf blade, PAR= thickness of parenchyma, EP=epidermis and ES= stomata. The bars represent 50 µm.

Fig 2. Adaxial surface (A to E) and abaxial (F through J) of maze leaves collected after a process of chromosome doubling with use of colchicine. Haploid material (A and F), diploid (B and G), Double haploid (C and H), triploid (D and I) and tetraploid (E and J). The bars represent 50 µm and arrows indicate stomata.

In Table 3, it is observed that the treatment 7 presented the highest values of ELF, ESS, EEI and held until the third position in the ranking for PR, DEI, DS. While the treatment 6 occupied until the second position to ELF, ESS, PR, DPS and DPI. The treatments 1 and 5 occupied the last two positions to ELF, EES, PR, EEI, DES, DPS, DEI and DPI.

Table 3. Genotypic averages and ranking of treatments for the anatomical characteristics related to leaf blade. Leaf blade thickness (ELF), thickness of the upper epidermis (ESS), thickness of the parenchyma (PAR), thickness of the lower epidermis (EEI), equatorial diameter of the stomata in the upper face (DES), polar diameter of the stomata in the upper face (DPS), polar and equatorial diameter of the stomata in the lower face (DEI) and (DPI), stomatal density of the upper surfaces (DS) and lower (DI) of maze leaves collected after a process of chromosome doubling with use of colchicine.

ELF		EES		PAR		EEI		DES	
Treat.	**$\overline{X}$ BLUP**	**Treat.**	**$\overline{X}$ BLUP**	**Treat.**	**$\overline{X}$ BLUP**	**Treat.**	**$\overline{X}$ BLUP**	**Treat.**	**$\overline{X}$ BLUP**
7	0.1375	7	0.0305	6	0.0858	7	0.0234	8	0.0378
6	0.1334	6	0.0284	7	0.0843	3	0.0215	3	0.0075
3	0.1237	2	0.0284	3	0.0761	8	0.0208	1	0.0075
8	0.1211	3	0.0284	8	0.0760	6	0.0189	4	0.0075
2	0.1112	8	0.0261	4	0.0664	2	0.0188	2	0.0075
4	0.1062	4	0.0249	2	0.0654	4	0.0173	7	0.0075
1	0.0957	5	0.0238	1	0.0621	1	0.0160	6	0.0075
5	0.0932	1	0.0218	5	0.0586	5	0.0155	5	0.0075
Mean	0.1152	-	0.0266	-	0.0719	-	0.0190	-	0.0113
DPS		**DEI**		**DPI**		**DS**		**DI**	
Treat.	**$\overline{X}$ BLUP**	**Treat.**	**$\overline{X}$ BLUP**	**Treat.**	**$\overline{X}$ BLUP**	**Treat.**	**$\overline{X}$ BLUP**	**Treat.**	**$\overline{X}$ BLUP**
8	0.0315	8	0.0433	8	0.0325	5	60.5125	5	110.2138

6	0.0270	3	0.0369	6	0.0284	1	51.5084	1	93.7531
3	0.0260	7	0.0326	3	0.0277	7	51.4221	4	93.4814
5	0.0238	6	0.0320	2	0.0240	6	51.2206	7	82.2563
7	0.0233	1	0.0300	5	0.0235	4	49.0210	6	78.8012
4	0.0233	2	0.0291	4	0.0235	3	46.3618	2	73.8440
2	0.0230	5	0.0288	7	0.0236	2	43.5809	3	70.7177
1	0.0213	4	0.0286	1	0.0214	8	37.8325	8	51.1355
Mean	0.0249	-	0.0326	-	0.0255	-	48.9325	-	81.7754

The importance or contribution of each analyzed variable as a possible marker in the separation of the tested plants, demonstrates greater relevance of the thickness of the leaf blade and the stomata, while the thickness of epidermis little contributed to the separation of plants. (Fig 3).

Fig 3. The relative importance of the characteristics evaluated in the separation of the valuated materials.

In parallel to the anatomical analyzes and with the aim of inferring the real ploidies observed in the tested materials, the DNA quantification was performed by flow cytometry technique in 32 selected plants (Table 4). The histograms obtained by this method allows for the identification of the ploidy level of the individuals tested through the location of the G1 peak of the sample on the axis of the relative intensity of fluorescence (Fig 4). The dominant peaks generated in the histograms are relative to the quantity of DNA of the cores in the G1 phase of the cell cycle. The estimate of the ploidy level is done by comparing the G1 peaks of the histogram of a sample with the peak of a plant-standard with known ploidy [25].

Maize diploids have the peak G1, located in the region of relative intensity of fluorescence, soon after the mark of 10^2 (Fig 4A). The haploids have lower relative intensity of fluorescence and the peak G1 is located to the left of the mark of 10^2, i.e., dislocated in the direction of the x axis (Fig 4B). Whereas the triploids and the tetraploids have still greater intensity than the diploids, locating at the right of the mark of 10^2 and with higher peaks (fig not shown). Whereas the Double haploid plants, are those that have ploidies haploid/diploid type (Fig 4C).

Usually, an association of the histogram obtained with a histogram of a standard plan is performed, for example, *Vicia faba,* used by [10,11,33]. The use of standard allows to obtain the quantity of DNA contained in the sample.

Fig 4. Histograms of ploidies detected by flow cytometry in maize plants collected after a process of chromosome doubling with use of colchicine. A. Diploid Plant B. Haploid Plant. C. Double haploid Plant. Vertical axis = number of read cores; horizontal axis = relative intensity of fluorescence. The arrows show the peaks G1 and G2 and the external standard of reference.

Table 4. Identification of the ploidy level in agreement with the analysis of flow cytometry and flow of 32 plants evaluated divided by treatment.

Treatments	Ploidies found
1	Haploid
	Haploid
	Haploid
	Double haploid
2	Haploid
	Diploid
	Diploid
	Diploid
3	Diploid
	Diploid
	Haploid
	Diploid
4	Haploid
	Diploid
	Diploid
	Haploid
5	Haploid
	Haploid
	Haploid
	Haploid
6	Diploid
	Diploid
	Diploid
	Diploid
7	Diploid
	Diploid
	Diploid
	Diploid
8	Triploid
	Triploid
	Diploid

Tetraploid

Discussion

It was possible through the analysis of mixed model to verify the existence of variability in the anatomical characteristics, i.e., there is a difference among the tested materials ($P < 0.01$) (Table 1). In addition, these variables showed a quality required for insertion in a breeding program, with the aim of separation of evaluated materials [34]. The heritability and accuracy obtained were high in accordance with the classification made by [31] (Table 1). The fact of the evaluated anatomical characteristics have potential for use in selection programs is of extreme importance, since these characteristics may be associated with the photosynthetic potential of plants, i.e., the productive capacity of plant material, leaves, roots and seeds.

The thickness of the leaf blade (ELF), has a crucial role not only in the capacity of carbon fixation by the chloroplasts of the palisade parenchyma, but also by the internal storage of CO_2 by sponge parenchyma [35]. While the stomata are the channels of influence of CO and the flow of water vapor. For the plants to be effective, they must balance the gaseous exchanges carried out through these structures to maximize the absorption of CO_2 for photosynthesis and minimize the loss of water through transpiration. Thus increasing the efficiency of the use of the water and consequently the plasticity of the plant in the face of environmental changes. A program that aims at obtaining hybrids with greater adaptive capacity, seems a major bottleneck of agriculture through the global climate changes [36].

The stomata behavior, therefore, controls the volume of CO_2 in the intercellular spaces of the leaf for photosynthesis. Even if the maze as plant of C4 metabolism is able through the mechanisms of CO_2 concentration, to maintain an adequate quantity of C for photosynthesis [37], the stomatal density and the size of the stomata are

344 important characteristics to maximize efficiency. Once that, in spite of the area of the
345 pores of the stomata represent less than 3% of the total area of the leaf, about 98% of
346 all the absorbed CO_2 and water lost occurs by these pores [38].

347 The anatomical characteristics of the stomata define the stomatal conductance
348 (*gs*), theoretical maximum [39], i.e., the functionality of the same and also influence the
349 speed of response. The *maximum gs* relates to the size and density of stomata, which
350 can be influenced by the environment of growth [40,41]. However, as in this study, all
351 the plants were grown in the same environment, we can consider that the density and
352 the pattern of size, influenced by the atmospheric concentration of CO_2, water
353 availability [42] and light [43], varied according to the genetic characteristics of each
354 tested hybrid. This reinforces the importance of the anatomical characters as early
355 markers of separation of hybrids used in this study.

356 Experimental evidences showed that the density of stomata is negatively
357 correlated with the stomata size [40,41]. The interaction/correlation among stomata
358 size and density, and the impact on stomatal function has received much attention,
359 particularly with reference to the evolution of the performance and plasticity in plants
360 [41]. Evidence from several studies have also suggested that smaller stomata respond
361 faster than larger stomata, an observation that has been explained in the context of
362 relations surface-volume and the requirement for ck to boost the movement [44].

363 The selection of plants grown with changes in the density of stomata to increase
364 the performance of plants has been widely exploited [38,45], with limited success. The
365 increase of the stomatal density can increase the *gs* and the photosynthetic rate can
366 become 30% greater in conditions of high brightness [46].

367 The increase of photosynthesis can encourage the increase in weight, as
368 already mentioned. Increase in the weight of seed has also been associated with
369 induced polyploidy [47]. What can potentiate the vigor and germination of seeds,
370 favoring the formation of a more homogeneous stand. However, the manipulation of

371 functional stomate responses is clearly more complicated, requiring a thorough
372 understanding of the metabolism of the manipulated plant.

373 The use of these anatomical characteristics of the leaf is widely used for
374 identifying the levels of ploidy in many species of plants, such as alfalfa [48],
375 *Gossypium* [49], *Dactylis* [50], ryegrass [51], wheat [52] and *Bromus inermis* [53,54]. In
376 coffee the density of stomata decreased while its size increased with an increase in the
377 ploidy level, with the lower density found in the tetraploids and higher in the diploids
378 [20]. Genotypic differences in stomatal frequency and length of the guard cells were
379 also observed in barley [55], soybean [56] Triticale [57,58]. These studies demonstrate
380 the possibility of the use of anatomical markers with mechanism for identification of
381 ploidy.

382 In the present, it is verified the contribution of the ten variables evaluated in
383 separate studied plants and coincides with what is reported in the literature in relation
384 to the great importance of the stomata. Carefully observing the relative contribution of
385 each trait, it is verified that the variables associated to the stomata represent,
386 altogether, approximately 50% of the contribution of separation (Fig 3).

387 The high contribution of leaf blade is due to its constitution. The leaf blade is
388 composed by parenchyma, in which chloroplasts and spaces for CO_2 storage are
389 located, in addition of course, all the other components of the leaf. Therefore its
390 relevance is easily understandable and the importance of variables related to the
391 stomata is also evident, reinforcing what is already described in several academic
392 articles.

393 In addition it is possible to suggest that the anatomic variables, as possible
394 markers were efficient on grouping even partially the hybrids (Tables 2 and 3).
395 Behavior that reinforces what has been described above, where the traits were efficient
396 anatomical markers for various crops. The treatments that showed higher averages for
397 the analyzed variables were classified as diploids (Table 3), in general with averages
398 exceeding the haploids (Table 2).

399 The increase in ploidy level may be the main driving force to facilitate the plants
400 breeding, as it provides important phenotypic effects, such as increasing the size of the
401 cells and organs, and sometimes a larger force and biomass, and additional molecular
402 and phenotypic variation that may arise soon after the formation of the polyploids. This
403 behavior can be attributed to the effect "gigantism", in which plants with higher ploidy
404 may have increased the size of their structures [19]. The treatments 6 and 7 (diploids),
405 for the characteristics related to leaf blade (ELF and PAR), showed higher averages to
406 the haploids (Table 2). However the effect "gigas" was not observed to hybrids tri and
407 tetraploids, as reported in *bulbophyllum ipanemense* [59].
408 Significant effects on the ploidy level, and the anatomical and morphological
409 characteristics, such as leaf dry mass and thickness of the epidermis, have already
410 been reported in Brassicas [60] and characteristics such as leaf thickness and
411 photosynthetic rate, for rice [61]. The increase in the leaves thickness and total mass of
412 plants may result in greater energy expenditure, however, as the maze is a plant of
413 Kranz anatomy, there is not so much spent on histodifferentiation of juxtaposed layers
414 of palisade parenchyma, since these cells are found around the cells of the sheath of
415 the beam. Therefore the gain in leaf thickness would contribute not only to the increase
416 of the total mass of the leaf, but also to the increase of empty spaces. These spaces
417 play an important role in the CO_2 reserve for photosynthesis and because it does not
418 require energy to histodifferentiation, being less costly in terms of energy.
419 The size of the cells and the thickness of the components were positively
420 correlated with the ploidy level also in potatoes [62]. With the increase of the genome,
421 the gigantism in cells and organs is widely observed and associated with the
422 increments in the photosynthetic rate [63]. The increase of photosynthesis is attributed
423 to the increase of the tissues, increased capacity for storage of CO_2, and increase of
424 *gs*. The size of the epidermal cells, cells, can also be associated with the ploidy level of
425 the material under observation [47]. In addition to the increase in the activity of multiple
426 enzymes such as hydrolases and expansins [64].

The effect "giga" was also related in previous studies with modifications of cell wall through a loosening, which enables a higher rate of growth of plants and phenotypic changes. This loosening is assigned to a higher expression of genes of expansin enzyme in rice [65,66], tobacco [67,68] and Arabidopsis [69,70]. The role of expansins would be to induce the extent of cell wall, generating larger cells, higher plants and longer roots. So in these cases, the cell expansion associated with the ploidy is related to the increase of molecular signaling for synthesis of genes of expansin and may lead to an increase in weight of structures, as in tomato [71].

Conclusions

The thickness of the leaf blade and the size of the stomata are highly heritable traits in maize.

The obtained high accuracy validates the anatomical tool through the variables studied in the present work, as a marker in the differentiation of ploidies in maize plants, which may be employed in programs for selection of hybrids.

The anatomy made in young leaves of maze is a non-destructible technique and in conjunction with the technique of flow cytometry, can be used as indirect method in programs to obtain double haploids, in the initial stage of identification of seedlings.

Conflicts of interest

The authors declare no conflict of interest.

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Author Contributions

Conceptualization: RMOP HOS EVRVP

Formal analysis: RMOP GAS

Funding acquisition: EVRVP

Investigation: RMOP GAS DRV RCCV

Writing: RMOP DRV RCCV

References

1. Bordes J, Charmet G, de Vaulx R., Pollacsek M, Beckert M, Gallais A. Doubled haploid vesus S1 family recurrent selection for testcross performance in a maize population. Theor Appl Genet. 2006;112: 1063–1072. doi:10.1007/s00122-006-0208-3
2. Melchinger AE, Schipprack W, Würschum T, Chen S, Technow F. Rapid and accurate identification of in vivo-induced haploid seeds based on oil content in maize. Sci Rep. 2013;3: 2129. doi:10.1038/srep02129
3. Prasanna BM, Chaikam V, Mahuku G. Doubled haploid technology in maize breeding: theory and practice. Texcoco: CIMMYT; 2012.
4. Chaikam V, Lopez LA, Martinez L, Burgueño J, Boddupalli PM. Identification of in vivo induced maternal haploids in maize using seedling traits. Euphytica. 2017;213: 177. doi:10.1007/s10681-017-1968-3
5. Ren R, Wu P, Tian X, Lübberstedt T, Chen S. QTL mapping for haploid male fertility by a segregation distortion method and fine mapping of a key QTL qhmf4 in maize. Theor Appl Genet. 2017;130: 1349–1359. doi:10.1007/s00122-017-2892-6
6. Eeckhaut TGR, Werbrouck SPO, Leus LWH, Van Bockstaele EJ,

476 Debergh PC. Chemically induced polyploidization in Spathiphyllum wallisii
477 Regel through somatic embryogenesis. Plant Cell Tissue Organ Cult.
478 2004;78: 241–246. doi:10.1023/B:TICU.0000025659.19232.04
479 7. Schifino-Wittmann MT, Dall´Agnol M. Indução de poliploidia no
480 melhoramento de plantas. Pesq Agrop Gaúcha. 2003;9: 155–164.
481 8. Dhooghe E, Van Laere K, Eeckhaut T, Leus L, Huylenbroeck JV. Mitotic
482 chromosome doubling of plant tissues in vitro. Plant Cell Tiss Organ Cult.
483 2011;104: 359–373. doi:10.1007/s11240-010-9786-5
484 9. Fritsche-Neto R, Garbuglio DD, Borém A. Duplo-haploides. Biotecnologia
485 aplicado ao melhoramento de plantas. Viçosa: UFV; 2012. pp. 267–301.
486 10. Battistelli G., Von Pinho RG, Justus A, Couto EG., Balestre M. Production
487 and identification of doubled haploids in tropical maize. Genet Mol Res.
488 2013;12: 4230–4242. doi:10.4238/2013.October.7.9
489 11. Couto EGO, Pinho EVRV, Pinho RGV, Carvalho MR, Bustamante FO,
490 Nascimento MS. Verification and characterization of chromosome
491 duplication in haploid maize. Genet Mol Res. 2015;14: 6999–7007.
492 doi:10.4238/2015.June.26.9
493 12. Dang NC, Munsch M, Aulinger I, Renlai W, Stamp P. Inducer line
494 generated double haploid seeds for combined waxy and opaque 2 grain
495 quality in subtropical maize (Zea mays. L.). Euphytica. 2012;183: 153–
496 160. doi:10.1007/s10681-011-0423-0
497 13. Lambers H, Chapin FS, Pons TL. Plant physiological ecology. New York:
498 Springer-Verlag; 2008. doi:10.1007/978-0-387-78341-3
499 14. Jokela A, Sarjala T, Huttunen S. The structure and hardening status of
500 Scots pine needles at different potassium availability levels. Trees.

1998;12: 490–498. doi:10.1007/s004680050179

15. Bosabalidis A., Kofidis G. Comparative effects of drought stress on leaf anatomy of two olive Cultivars. Plant Sci. 2002;163: 375–379. doi:https://doi.org/10.1016/S0168-9452(02)00135-8

16. Oksanen EJ. Increasing tropospheric ozone level reduced birch (Betula pendula) dry mass within a five years period. Water, Air Soil Pollut. 2001;130: 947–952. doi:https://doi.org/10.1007/978-94-007-0810-5_5

17. Oksanen E, Häikiö E, Sober J, Karnosky D. Ozone-induced H2O2 accumulation in field-grown aspen and birch is linked to foliar ultrastructure and peroxisomal activity. New Phytol. 2004;161: 791–799. doi:https://doi.org/10.1111/j.1469-8137.2003.00981.x

18. Ramalho MAP, Santos JB, Pinto CABP. Genética na agropecuária. Lavras: UFLA; 2012.

19. Vichiato MRV, Vichiato M, Moacir P, Castro DM, Dutra LF. Indução e identificação de tetraplóides em Dendrobium nobile Lindl. (Orchidaceae). Rev Ciênc Agron. 2007;38: 385–390.

20. Mishra MK. Stomatal Characteristics at Different Ploidy Levels in Coffea L. Ann Bot. 1997;80: 689–692. doi:https://doi.org/10.1006/anbo.1997.0491

21. Padoan D, Mossad A, Chiancone B, Germana MA, Khan PSSV. Ploidy levels in Citrus clementine affects leaf morphology, stomatal density and water content. Theor Exp Plant Physiol. 2013;25: 283–290. doi:http://dx.doi.org/10.1590/S2197-00252013000400006

22. Rocha S. Caracterização de híbridos triploides espontâneos de citros. Instituto Agronômico de Campinas. 2014.

526 23. Couto EGO, Pinho EVRV, Pinho RGV, Veiga AD, Bustamante FO, Dias
527 KOG. In vivo haploid induction and efficiency of two chromosome
528 duplication protocols in tropical maize. Ciênc e Agrotec. 2015;39: 435–
529 442. doi:http://dx.doi.org/10.1590/S1413-70542015000500002
530 24. Chase SS, Nanda DK. Comparison of variability in inbred lines and
531 monoploid-derived lines of maize (Zea mays L.). Crop Sci. 1965;5: 275–
532 276.
533 25. Dolezel J. Current topics in plant cytogenetics related to plant
534 improvement. Current topics in plant cytogenetics related to plant
535 improvement. Vienna: WUV-Universitätsverlag; 1997. pp. 80–90.
536 26. Johansen DA. Plant microtechnique. New York: McGraw-Hill; 1940.
537 27. O'Brien T, Feder N, McCully ME. Polychromatic staining of plant cell walls
538 by toluidine blue. O Protoplasma. 1964;59: 367–373.
539 doi:10.1007/BF01248568
540 28. Zsögön A, Negrini ACA, Peres LEP, Nguyen HT, Ball MC. A mutation that
541 eliminates bundle sheath extensions reduces leaf hydraulic conductance,
542 stomatal conductance and assimilation rates in tomato (Solanum
543 lycopersicum). New Phytol. 2014;205: 618–626. doi:10.1111/nph.13084
544 29. Resende MDV. Software SELEGEN – REML/BLUP: Sistema estatistico e
545 seleção computadorizada via modelos lineares mistos. Colombo:
546 EMBRAPA Florestas; 2007.
547 30. Resende MDV de. Matemática e estatística na análise de experimentos e
548 no melhoramento genético. Colombo: Embrapa Florestas; 2007.
549 31. Resende MDV, Duarte JB. Precisão e controle de qualidade em
550 experimentos de avaliação de cultivares. Pesq Agropec Trop. 2007;37:

551 182–194.

552 32. Falconer DS, Mackay TFC. Introduction to quantitative genetics. Harlow:
553 Burnt Mill; 1996.

554 33. Ribeiro CB. Estratégias para obtenção de duplo-haploides e progênies
555 indutoras de haploidia em milho. Universidade Federal de Lavras. 2016.

556 34. Silva MR, Pinheiro RV, Christoforo AL, Panzerad TH, Lahr FAR. Hybrid
557 Sandwich Particleboard Made with Sugarcane, Pínus Taeda Thermally
558 Treated and Malva Fibre from Amazon. Mater Res. 2017;21.
559 doi:http://dx.doi.org/10.1590/1980-5373-mr-2017-0724.

560 35. Martins SVC, Galmés J, Cavatte PC, Pereira LF, Ventrella MC, DaMatta
561 FM. Understanding the low photosynthetic rates of sun and shade coffee
562 leaves: Bridging the gap on the relative roles of hydraulic, diffusive and
563 biochemical constraints to photosynthesis. PLoS One. 2014;9: e95571.
564 doi:https://doi.org/10.1371/journal.pone.0095571

565 36. Cochrane JA, Hoyle GL, Yates CJ, Wood J, Nicotra AB. Evidence of
566 population variation in drought tolerance during seed germination in four
567 Banksia (Proteaceae) species from Western Australia. Aust J Bot.
568 2014;62: 481–489. doi:10.1071/BT14132

569 37. Taiz L, Zeiger E. Plant Physiology. 5th ed. Sunderland: Sinauer
570 Associates; 2015.

571 38. Lawson T, Blatt MR. Stomatal size, speed, and responsiveness impact on
572 photosynthesis and water use efficiency. Plant Physiol. 2014;164: 1556–
573 1570. doi:https://doi.org/10.1104/pp.114.237107

574 39. Dow GJ, Bergmann DC, Berry JA. An integrated model of stomatal
575 development and leaf physiology. New Phytol. 2014;201: 1218–1226.

doi:10.1111/nph.12608

40. Hetherington AM, Woodward FI. The role of stomata in sensing and driving environmental change. Nature. 2003;424: 901–908. doi:10.1038/nature01843

41. Franks PJ, Farquhar GD. The mechanical diversity of stomata and its significance in gas-exchange control. Plant Physiol. 2007;143: 78–87. doi:https://doi.org/10.1104/pp.106.089367

42. Gray JE, Holroyd GH, Van der Lee FM, Bahrami AR, Sijmons PC, Woodward FI, et al. The HIC signalling pathway links CO_2 perception to stomatal development. Nature. 2000;408: 713–716. doi:10.1038/35047071

43. Gay AP, Hurd RG. The influence of light on stomatal density in the tomato. New Phytol. 1975;75: 37–46. doi:https://doi.org/10.1111/j.1469-8137.1975.tb01368.x

44. P.L. D, Froend RH, Franks PJ. Smaller, faster stomata: scaling of stomatal size, rate of response, and stomatal conductance. J Exp Bot. 2013;64: 495–505. doi:10.1093/jxb/ers347

45. Jones HG. Transpiration in barley lines with differing stomatal frequencies. J Exp Bot. 1977;23: 162–168. doi:https://doi.org/10.1093/jxb/28.1.162

46. Schluter U, Muschak M, Berger D, Altmann T. Photosyntetic performance of an Arabidopsis mutant with elevated stomatal density (sdd1-1) under different light regimes. J Exp Bot. 2003;54: 867–874. doi:10.1093/jxb/erg087

47. Yoon S, Aucar S, Hernlem BJ, Edme S, Palmer N, Sarath G, et al.

Generation of Octaploid Switchgrass by Seedling Treatment with Mitotic Inhibitors. BioEnergy Res. 2017;10: 344–352. doi:https://doi.org/10.1007/s12155-016-9795-2

48. Bingham ET. Stomatal chloroplasts in alfalfa at four ploidy levels. Crop Sci. 1968;8: 509–510. doi:10.2135/cropsci1968.0011183X000800040036x

49. Krishnaswami R, Andal R. Stomatal chloroplast number in diploids and polyploids of Gossypium. Proc Indian Acad Sci. 1978;87: 109 – 112. doi:https://doi.org/10.1007/BF03046960

50. Santen EV, Casler EV. Evaluation of indirect ploidy indicators in Dactylis C. subspecies. Crop Sci. 1986;26: 848–852.

51. G. J. S, Post Jr J, Dijkstra H. The length of stomata as an indicator for polyploidy in rye-grasses. Euphytica. 1965;14: 225–230. doi:https://doi.org/10.1007/BF00149503

52. Wang ZY, Khan WA, Bickers DR, Mukhtar H. Protection against polycyclic aromatic hydrocarbon-induced skin tumor initiation in mice by green tea polyphenols. Carcinogenesis. 1989;10: 411–415. doi:10.1093/carcin/10.2.411

53. Tan GY, Dunn G. Relationship of stomatal lenght and frequency and pollen grain diameter to ploidy level in Bromus inermis leyss. Crop Sci. 1973;13: 332–334. doi:10.2135/cropsci1973.0011183X001300030014x

54. Lea HZ, Dunn GM, Koch DW. Stomatal diffusion resistance in three ploidy levels of smooth bromegrass. Crop Sci. 1977;17: 91–93. doi:10.2135/cropsci1977.0011183X001700010026x

55. Mishkin MK, Rasmussen DC. Frequency and distribution of stomata in

626 barley. 1970. 8AD;10: 575–578.
627 doi:doi:10.2135/cropsci1970.0011183X001000050038x
628 56. Chia AJ, Brun WA. Stomatal size and frequency in soybeans. Crop Sci.
629 1975; 309–313. doi:10.2135/cropsci1975.0011183X001500030008
630 57. Teare ID, Peterson CJ, Law AG. Size and frequency of leaf stomata in
631 cultivars of Triticum aestivum and other Triticum species. Crop Sci.
632 1971;11: 496–498. doi:10.2135/cropsci1971.0011183X001100040010x
633 58. Sapra VT, Hughes JL, Sharma GC. Frequency, size and distribution of
634 stomata in triticale leaves. Crop Sci. 1975;15: 356–358.
635 doi:10.2135/cropsci1975.0011183X001500030022x
636 59. Farinaci JS. Variabilidade genética em algumas espécies de
637 Bulbophyllum Thouars (Orchidaceae) de campos rupestres. Universidade
638 Estadual de Campinas. 2001.
639 60. Baker RL, Yarkhunova Y, Vidal K, Ewers BE, Weinig C. Polyploidy and
640 the relationship between leaf structure and function: implications for
641 correlated evolution of anatomy, morphology, and physiology in Brassica.
642 BMC Plant Biol. 2017;17. doi:10.1186/s12870-016-0957-3
643 61. Giuliani R, Koteyeva N, Voznesenskaya E, Evans MA, Cousins AB,
644 Edwards GE. Coordination of leaf photosynthesis, transpiration, and
645 structural traits in rice and wild relatives (genus Oryza). Plant Physiol.
646 2013;162: 1632–1651. doi:https://doi.org/10.1104/pp.113.217497
647 62. Stupar RM, Bhaskar PB, Yandell BS, Rensink WA, Hart AL, Ouyang S, et
648 al. Phenotypic and transcriptomic changes associated with potato
649 autopolyploidization. Genetics. 2007;176: 2055–2067.
650 doi:https://doi.org/10.1534/genetics.107.074286

651 63. Zhou Y, Kang L, Liao S, Pan Q, Ge X, Li Z. Transcriptomic analysis
652 reveals differential gene expressions for cell growth and functional
653 secondary metabolites in induced autotetraploid of Chinese woad (Isatis
654 indigotica Fort.). PLoS One. 2015;10: e0116392.
655 doi:https://doi.org/10.1371/journal.pone.0116392
656 64. Warner DA, Ku MS, Edwards GE. Photosynthesis, leaf anatomy, and
657 cellular constituents in the polyploid C(4) grass Panicum virgatum. Plant
658 Physiol. 1987;84: 461–466. doi:10.1104/pp.84.2.461
659 65. Lee Y, Choi D. Biochemical Properties and Localization of the β-Expansin
660 OsEXPB3 in Rice (Oryza sativa L.). Mol Cells. 2005;20: 119–126.
661 doi:10.1002/047001539X.ch7
662 66. Ma N, Wang Y, Qiu S, Kang Z, Che S, Wang G, et al. Overexpression of
663 Os EXPA8, a root-specific gene, improves rice growth and root system
664 architecture by facilitating cell extension. PLoS One. 2013;8: e75997.
665 doi:https://doi.org/10.1371/journal.pone.0075997
666 67. Li F, Han Y, Feng Y, Xing S, Zhao M, Chen Y, et al. Expression of wheat
667 expansin driven by the RD29 promoter in tobacco confers waterstress
668 tolerance without impacting growth and development. J Biotechnol.
669 2013;163: 281–291. doi:10.1016/j.jbiotec.2012.11.008
670 68. Xu Q, Xu X, Shi Y, Xu J, Huang B. Transgenic tobacco plants
671 overexpressing a grass PpEXP1 gene exhibit enhanced tolerance to heat
672 stress. PLoS One. 2014;9: e100792.
673 doi:https://doi.org/10.1371/journal.pone.0100792
674 69. Hu Y, Zhu N, Wang X, Yi Q, Zhu D, Lai Y, et al. No TitleAnalysis of rice
675 Snf2 family proteins and their potential roles in epigenetic regulation.

Plant Physiol Biochem. 2013;70: 33–42. doi:10.1016/j.plaphy.2013.05.001

70. Goh H, Sloan J, Malinowski R, Fleming A. Variable expansin expression in Arabidopsis leads to different growth responses. J Plant Physiol. 2014;171: 329–339. doi:10.1016/j.jplph.2013.09.009

71. Cheniclet C, Rong WY, Causse M, Frangne N, Bolling L, Carde JP, et al. Cell expansion and endoreduplication show a large genetic variability in pericarp and contribute strongly to tomato fruit growth. Plant Physiol. 2005;139: 1984–1994. doi:https://doi.org/10.1104/pp.105.068767

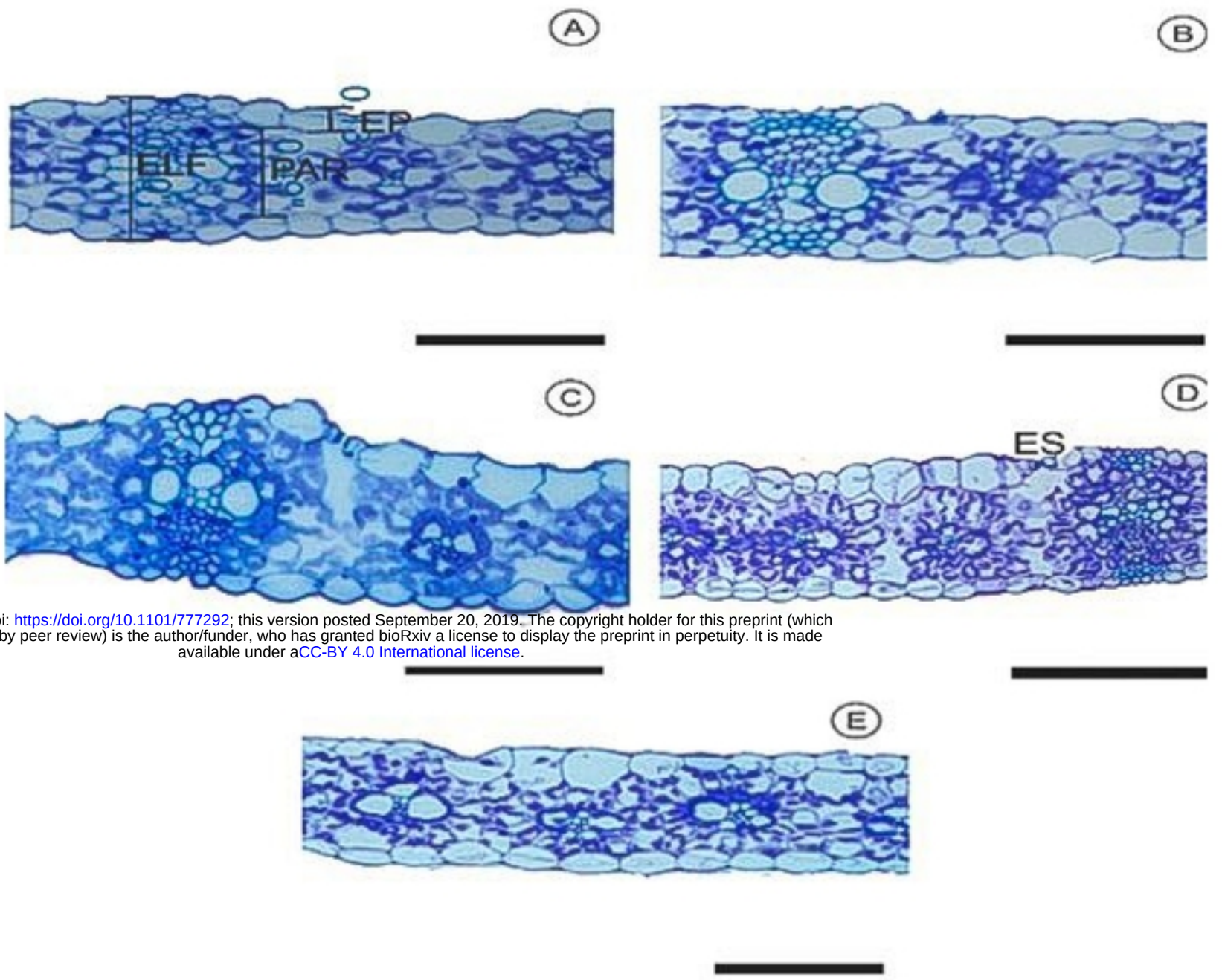

Fig 1. Cross-sectional cuts of maze leaves collected after a process of chromosome doubling with use of colchicine. Haploid material (A), diploid (B), Double haploid (C), triploid (D) and tetraploid (E). ELF= thickness of leaf blade, PAR= thickness of parenchyma, EP=epidermis and ES= stomata. The bars represent 50 μm.

Fig 1

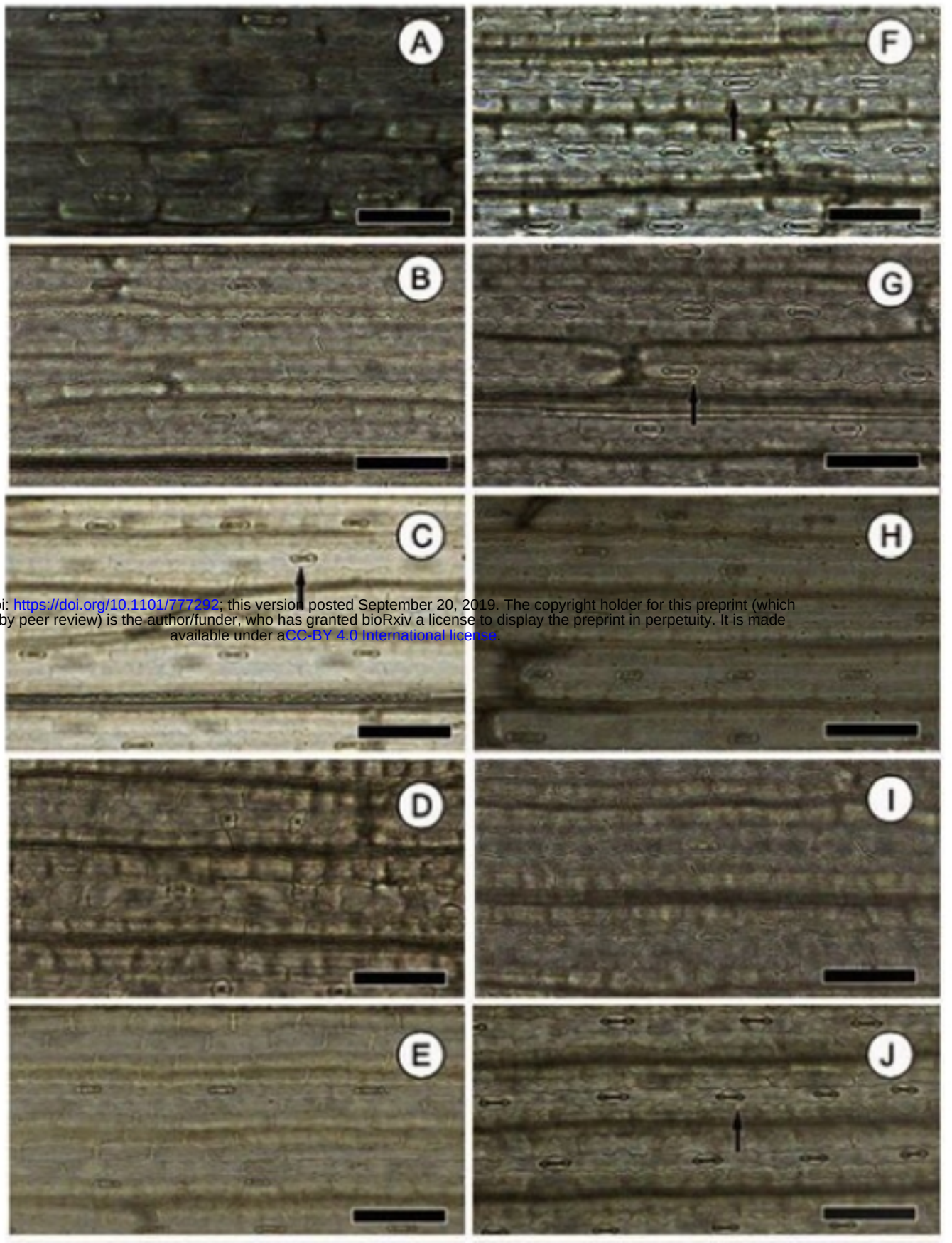

Fig 2. Adaxial surface (A to E) and abaxial (F through J) of maze leaves collected after a process of chromosome doubling with use of colchicine. Haploid material (A and F), diploid (B and G), Double haploid (C and H), triploid (D and I) and tetraploid (E and J). The bars represent 50 μm and arrows indicate stomata.

Fig 2

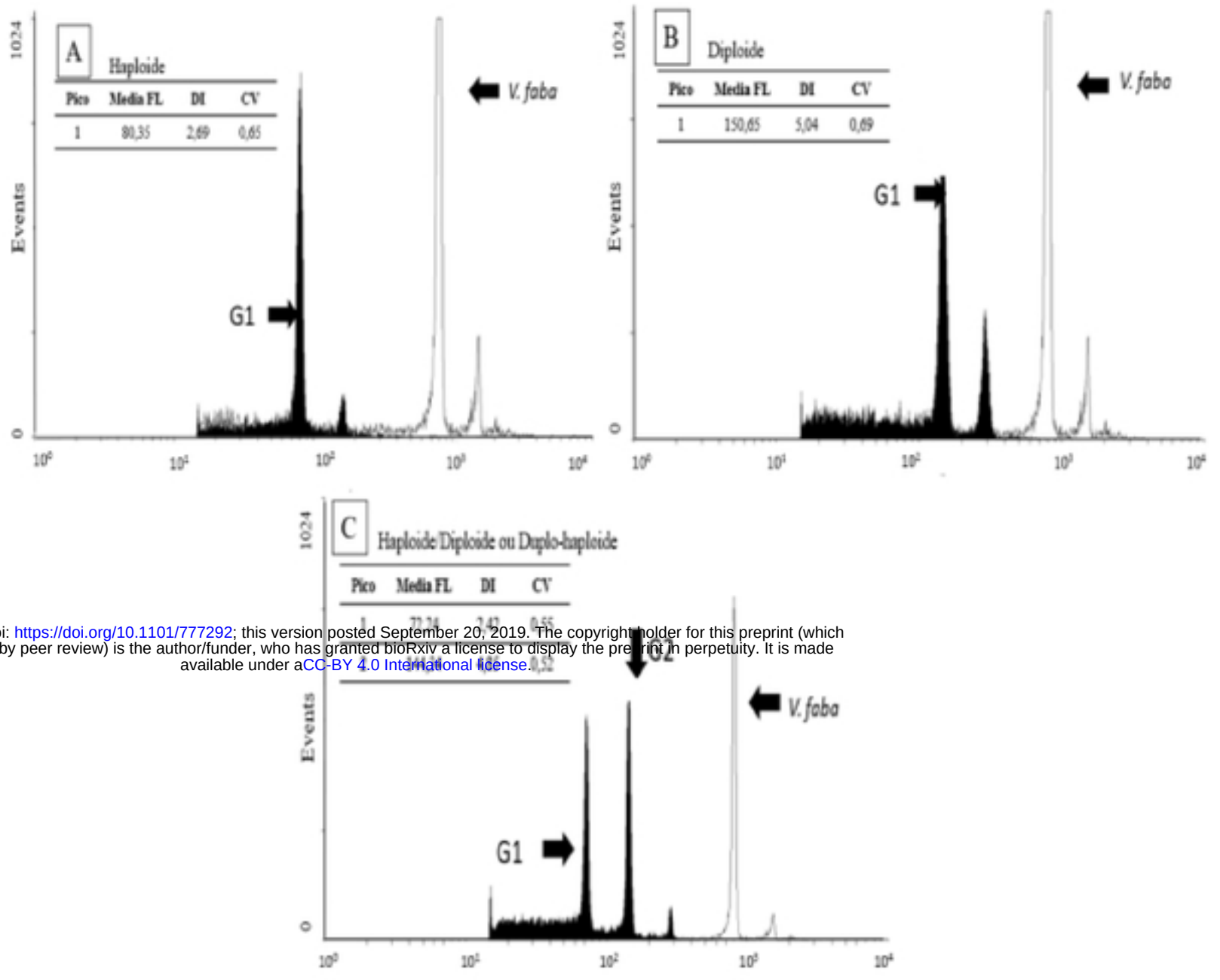

Fig 3. The relative importance of the characteristics evaluated in the separation of the valuated materials.

Fig 3