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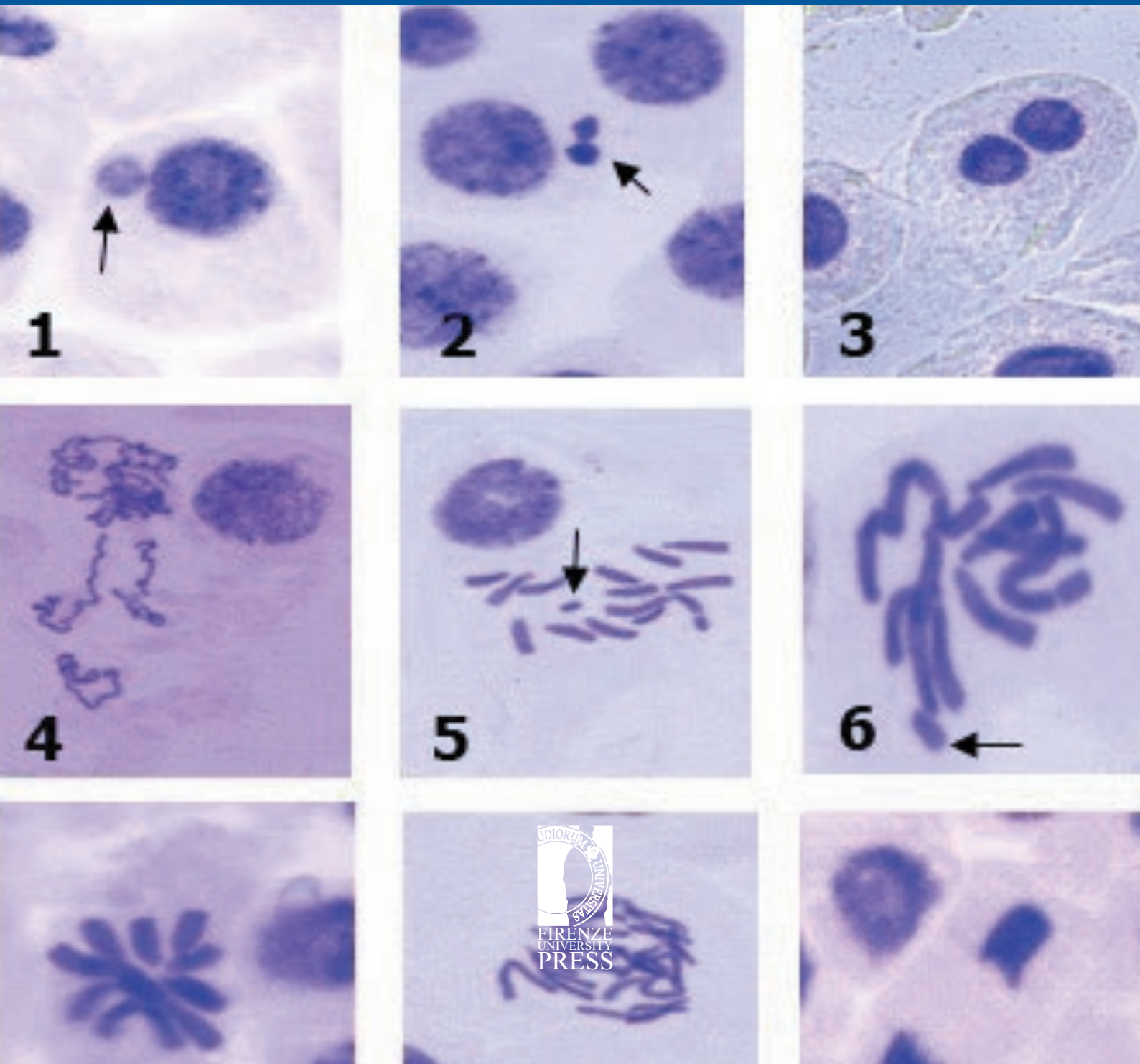

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Cover: figure from the article inside by Rodríguez-Domínguez et al. "Physical mapping of 45S and 5S rDNA in two *Sprekelia formosissima* cytotypes (Amaryllidaceae) through Fluorescent *In Situ* Hybridization (FISH)". Karyogram of diploid *Sprekelia formosissima*.

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Cytogenetic study of the *Bison bonasus*; I: Identification of heterochromatic regions and NORs in European bison karyotype and comparison with domestic cattle (*Bos taurus*)

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Abstract. The karyotypes of European bison *Bison bonasus* and domestic cattle *Bos taurus* are characterized by a diploid number of chromosomes, 2n = 60. Here, we characterized the European bison karyotype in terms of size and distribution of constitutive heterochromatin blocks (C-bands) and the location and number of nucleolar organizer regions (NORs), results were compared with those obtained for domestic cattle. For this purpose, staining for C and NOR bands was performed. In the chromosomes of both species, C-bands were located in the centromeric region of all chromosomes analyzed, except for the X chromosome. Active NORs in European bison chromosomes were identified in the chromosomes from pairs 2, 3, 4, 25, and 28. In cattle, NORs were located in chromosomes from pairs 2, 3, 4, 11, and 25. The average number of NORs in the cell of European bison and cattle was 4.47 ± 1.74 and 4.56 ± 1.66, respectively. The obtained results shed new light on the European bison cytogenetics and confirmed the high similarity between studied species.

Keywords: *Bison bonasus*, *Bos taurus*, heterochromatin, nucleolar organizer regions, nor, c-banding.

INTRODUCTION

The identification of homologous chromosomes is carried out on the basis of different banding techniques that reveal specific regions of the chromosome, for example constitutive heterochromatin (Sumner 1972) or Nucleolar Organizer Regions (NOR) (Goodpasture and Bloom 1975). The differentiated NOR sizes within a homology pair and the variability of constitutive heterochromatin blocks are treated as a chromosomal polymorphism, and variants that differ in NOR or C-bands are considered as chromosomal markers.

Constitutive heterochromatin is visible in the form of C-bands, is a late replicating fraction and contains a small number of genes (Lawce 2017).

Moreover, it was found that the DNA found in the constitutive heterochromatin blocks is a region that does not express genetic expression (Brown 1966). Methylation within CpG islands affects the significant level of condensation of constitutive heterochromatin (Spector 2003). Heterochromatin is also involved in the transcription and segregation of chromosomes (Grewal and Jia 2007).

Sipko et al. (2004) published a study in which they compared the karyotypes of *Bison bonasus* and *Bos taurus* by using the Sumner (1972) staining method of C-banding and SCE (sister chromatid exchange). They found no difference between the chromosomes of both species. The C-band method used by the authors showed that heterochromatic blocks were identified in all chromosomes except the X chromosome in both species.

The nucleolar organizer regions usually form secondary constrictions and are the localization site for genes encoding ribosomal nucleic acids (rRNA). NORs are organized as blocks of tandem repeating units whose distribution, i.e. the number of chromosomal loci and the number of genes in each locus, is a constant and characteristic for a given species (Weisenberger and Scheer 1995). Due to the affinity of NOR for heavy metals, nucleolar organizer regions appear in the form of black silver stripes during the dyeing process. Silver staining does not allow revealing all areas containing rRNA genes, but only active areas for nucleus formation in interphase (Verma and Babu 1995).

Graphodatsky et al. (1990) using classic staining method identified NORs on 2nd, 3rd, 4th and 28th chromosome pairs, while Gallagher et al. (1999) used *in situ* hybridization to identify NORs in *Bison bonasus* chromosomes and observed signals on chromosomes from pairs 2, 3, 11, 25, and 28.

Because of the limited amount of available literature, there is still much scope to discover and describe the cytogenetics of the species *Bison bonasus* also from the standpoint of separately maintained two lines of European bison: Lowland and Lowland-Caucasian (Pucek et al. 2004).

The aim of the study was to characterize the European bison karyotype in terms of size and distribution of constitutive heterochromatin blocks as well as the location and number of nucleolar organizer regions. It was decided to compare the karyotypes of the two studied species and confirm their similarity. Furthermore, we decided to confirm the location of NORs in the European bison. This is the first study in which the size of the heterochromatin area was measured in European bison.

MATERIALS AND METHODS

The experimental material consisted of 12 blood samples collected from *Bison bonasus* males from four localities (Figure 1, Table 1). The samples were stored in 9 ml heparinized tubes (Medlab Products) to prevent blood clotting and stored in cold until laboratory analysis. Blood from domestic cattle bull (*Bos taurus*) obtained in abattoir was used as a comparative material.

Cell culture

The cultures were carried out in 15 ml falcon tubes containing 8.5 ml of the culture medium RPMI 1640

Figure 1. Collection sites of European bison (*Bison bonasus*) samples in Poland. The numbering of sampling localities corresponds to the data in the Table 1.

Table 1. Collection sites of European bison (*Bison bonasus*) samples in Poland. The numbering of sampling localities corresponds to the Figure 1. SM – submetacentric, A – acrocentric.

Locality	Latitude, longitude	No. of specimens	2n	X	Y
Niepołomice	50°02'29.6"N, 20°21'54.1"E	3	60	SM	A
Gołuchów	51°51'35.2"N, 17°55'28.6"E	3	60	SM	A
Białowieża	52°42'20.0"N, 23°47'46.0"E	3	60	SM	A
Muczne	49°08'32.1"N, 22°42'50.5"E	3	60	SM	A

(SIGMA) with addition of 10% fetal bovine serum (SIGMA), pokeweed mitogen (SIGMA), and antibiotic (penicillin 100 µg/mL and streptomycin 100 µg/mL)(SIGMA). A thoroughly mixed blood sample was added to the final volume of 10 ml. The cultures were carried out in duplicate for each individual. The tubes were incubated at 38.5 °C for 72 hours and regularly mixed twice a day. The cultures were treated for 1 hour by colchicine (0.01 µg/mL) (SIGMA) (added at 71 hours). Subsequently, the cultures were treated with hypotonic solution (0.05 M KCl (POCH)) for 20 minutes at 38.5 °C, and then three times fixed in freshly prepared freezing cold Carnoy's fixative (POCH).

Microscope slide preparation

Slides were prepared before the staining procedures. The mixture of the fixed precipitate was spotted on a microscope slide and then air dried.

C-banding and AgNOR staining

Initially, the slides were subjected to the standard staining procedure described by Sumner (1972). The results obtained in the form of poorly visible C-bands were unsatisfactory; hence, we decided to use the methodology described by Chaves et al. (2000). Silver staining was carried out according to the method described by Howell and Black (1980).

Analysis of the slide preparations

By using a Nikon Eclipse 90i microscope connected to the DS5-U1 digital camera (Nikon Corporation, Tokyo, Japan), and a Zeiss Axiophot fluorescence microscope and LUCIA software (Laboratory Imaging Ltd, Prague, Czech Republic), 25 clearly visible and well dispersed metaphase plates were photographed for each male of both species. The measurements were made in the IMAGEJ program by using the LEVAN plugin (Wayne Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, USA, 2018). The obtained results were characterized statistically.

RESULTS AND DISCUSSION

The constitutive heterochromatin is a fraction which is most often located near the centromere of the chromosome, but it is also located in the distal parts of the chromosome arms, sometimes between the centromere and the telomeres or occupies the entire chromosomal arms (Lawce 2017). In our own research we measured the block size of constitutive heterochromatin in relation to the entire length of the arms of all analyzed chromosomes. The used procedure allowed us to determine the position of C-bands on European bison and domestic cattle chromosomes, and thus to identify sex chromosomes of both species (Figure 2A and 3A, respectively). The C-bands were located in the centromeric regions of all autosomes in both species and on Y chromosomes. In both species, no positive band was identified on X chromosomes. A positive, small, and dark band was found at the end of the short arm of the smallest chromosome of *Bison bonasus*, the Y chromosome. This chromosome is largely heterochromatic. Further, on the Y chromosome of domestic cattle, a dark and distinct positive band located at the end of the shorter arm was visible. By comparing the image obtained for the Y chromosome of European bison and domestic cattle, the difference in their morphology can be seen. The Y chromosome of domestic cattle is submetacentric, while that of the European bison is a small acrocentric as reported (Graphodatsky et al. 1990). Originally, the European bison's Y chromosome was considered submetacentric (Fedyk and Sysa 1971). In both species, 59 blocks of constitutive heterochromatin were identified on the 30 pair of chromosomes (Figure 2B and 3B).

In the analyzed acrocentric autosomes of European bison (Table 2) and domestic cattle (Table 3), a clear differentiation in the extent of heterochromatin areas was observed. In the measurement of constitutive heterochromatin block size in relation to the entire length of the European bison chromosome, the first pair of chromosomes showed the smallest mean value (14.52%), and the twelfth pair of chromosomes (26.40%) showed the highest value. In cattle, the first pair of chromosomes showed the smallest mean value (14.06%), while the twenty-third pair showed the highest value (32.50%). There were no positive blocks of constitutive heterochromatin on the X chromosomes in both species. The heterochromatin content of the Y chromosome of European bison and cattle was 39.15% and 28.95%, respectively. This indicates that the European bison's Y chromosome is much more heterochromatic. The variable length of heterochromatin blocks does not affect the phenotype, however it differentiates the morphology of the chromosomes of a given species. It has been found that the variable width of positive C-banding blocks indicates the presence of non-identical heterochromatin size (McFeely 1990).

The number of active NORs and their location in chromosomes is a characteristic of each species. In the

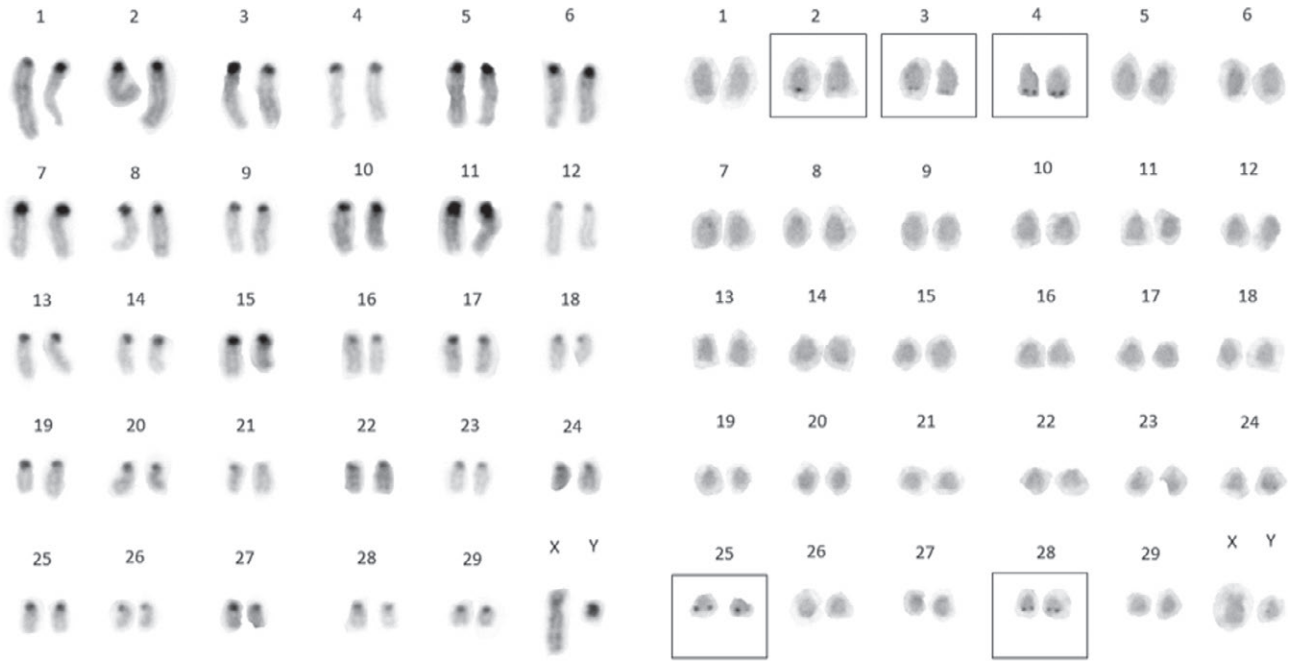

Figure 2. Karyogram of European bison male (*Bison bonasus*), (a) C-banding, (b) AgNOR-banding. Heterochromatic blocks are mainly located in centromeric positions of all autosomes and in sex chromosomes. NORs are located in 2, 3, 4, 25 and 28 chromosome pairs (marked in squares).

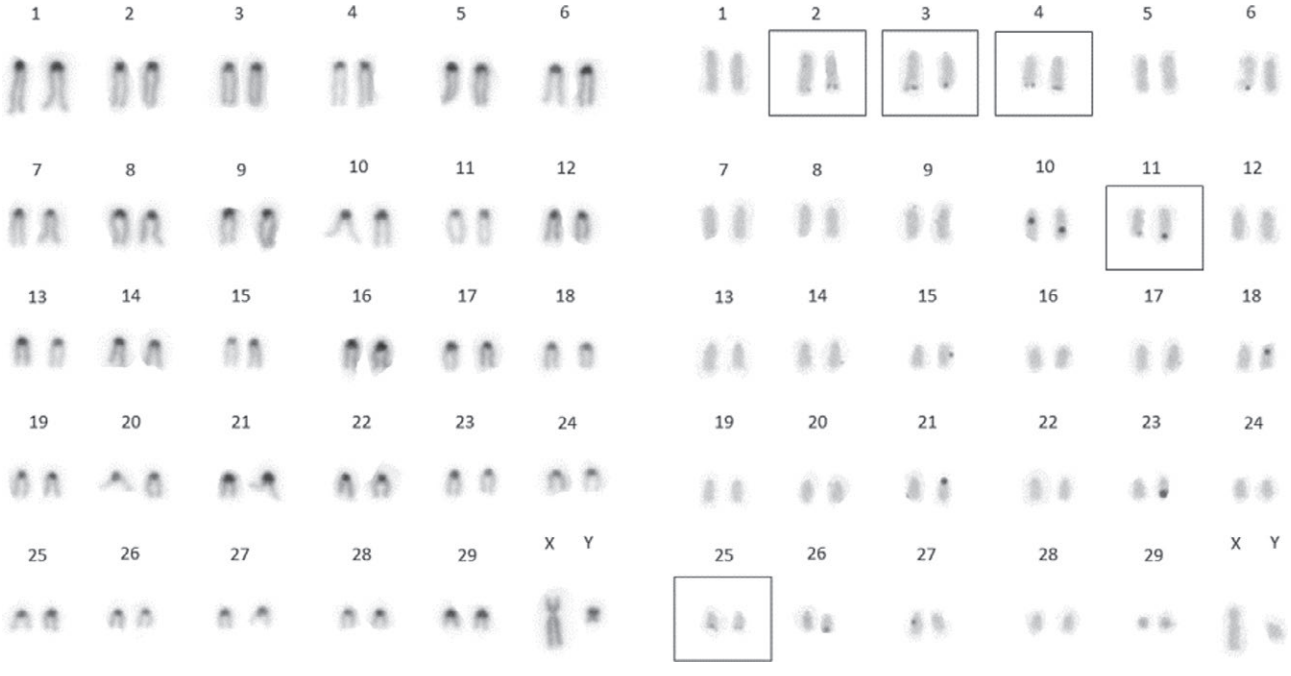

Figure 3. Karyogram of domestic cattle male (*Bos taurus*), (a) C-banding, (b) AgNOR-banding. Heterochromatic blocks are located in centromeric positions of all autosomes and in sex chromosomes. NORs are located in 2, 3, 4, 11 and 25 chromosome pairs (marked in squares).

metaphases of the same individual, there is an intercellular diversity of expression of NOR, which refers to the amount, size and intensity of silver staining (Weisenberger and Scheer 1995). The AgNOR staining enabled to identify active nucleating regions (NORs) found on the chromosomes of European bison and domestic cattle

Table 2. Size of constitutive heterochromatin blocks in European bison (*Bison bonasus*) chromosomes.

Chromosomes 1-14; XY	Average area of the constitutive heterochromatin $\bar{x} \pm S$	Chromosomes 15-29	Average area of the constitutive heterochromatin $\bar{x} \pm S$
1	14.52 ± 5.23	15	20.64 ± 2.24
2	16.49 ± 2.29	16	23.64 ± 12.46
3	17.78 ± 4.17	17	22.40 ± 0.89
4	16.05 ± 5.86	18	23.48 ± 1.68
5	25.06 ± 4.08	19	22.47 ± 0.70
6	23.95 ± 1.66	20	20.38 ± 1.84
7	16.79 ± 1.52	21	19.22 ± 1.77
8	18.80 ± 4.22	22	22.64 ± 3.04
9	21.33 ± 3.20	23	22.01 ± 7.13
10	23.68 ± 2.25	24	23.93 ± 2.16
11	18.12 ± 0.80	25	25.55 ± 3.41
12	26.40 ± 0.26	26	18.04 ± 6.04
13	17.18 ± 1.89	27	21.77 ± 3.64
14	21.14 ± 3.82	28	21.92 ± 2.21
X	-	29	21.11 ± 3.14
Y	39.15 ± 1.20		

Table 3. Size of constitutive heterochromatin blocks in cattle (*Bos taurus*) chromosomes.

Chromosomes 1-14; XY	Average area of the constitutive heterochromatin $\bar{x} \pm S$	Chromosomes 15-29	Average area of the constitutive heterochromatin $\bar{x} \pm S$
1	14.06 ± 1.60	15	19.95 ± 1.76
2	18.46 ± 3.15	16	18.39 ± 1.15
3	17.66 ± 1.40	17	23.11 ± 2.91
4	16.80 ± 2.51	18	25.19 ± 5.46
5	15.35 ± 1.87	19	25.51 ± 5.70
6	17.73 ± 3.63	20	26.69 ± 4.12
7	15.41 ± 4.47	21	32.15 ± 4.26
8	18.01 ± 1.13	22	30.98 ± 0.92
9	16.67 ± 2.53	23	32.50 ± 0.76
10	21.72 ± 4.86	24	21.47 ± 4.35
11	20.50 ± 3.35	25	27.57 ± 5.43
12	20.22 ± 2.44	26	25.54 ± 1.28
13	23.04 ± 3.21	27	25.77 ± 2.02
14	27.67 ± 2.80	28	24.56 ± 0.30
X	-	29	27.78 ± 6.29
Y	28.95 ± 9.34		

(Figure 2B and 3B, respectively). In European bison, the active nuclear regions were found in the terminal parts of chromosomes from pairs 2, 3, 4, 25, and 28 (Figure 2B). Similar conclusions were made by Gallagher et al. (1999) by using *in situ* hybridization for this purpose. In domestic cattle, visible silver grains were observed on chromosomes from pairs 2, 3, 4, 11, and 25 (Figure 3B). In a total of 325 metaphase plates analyzed (300 for European bison and 25 for domestic cattle) (Table 4), 1476 active nucleolar regions were observed (Table 5). There were visible differences in the intensity of NORs on chromosomes and the differences in the minimum and maximum number of active NORs in cells. At the cellular level, one to eight active NORs were observed in *Bison bonasus* species with an average value of 4.47 ± 1.75. In the *Bos taurus* species, it ranged from two to eight active nuclear regions with an average value of 4.56 ± 1.66. For comparison, in a similar experiment, the obtained active NORs average value for cattle was 6.06 (Mayr et al. 1987). The number of NORs in the cells of the animals varied and ranged from 107 to 115 within the individual. The most frequently observed cells had four active NORs, and the least frequently observed cells had eight.

Our research allowed us to characterize the European bison karyotype in terms of the C-band pattern and the number and location of active NORs. The following conclusions were made on the basis of the analyzes carried out. The number of active NORs per cell differed between the tested individuals and between the cells of the same individual. The method used in this study to identify C-bands can be successfully used to identify sex chromosomes. Constitutive heterochromatin has been identified in all European bison (*Bison bonasus*) and domestic cattle (*Bos taurus*) chromosomes, except for the X chromosome in both species. Chromosomes of both species can be compared on the basis of different classical staining methods, but a thorough analysis aimed at comparison of the Y chromosome requires more advanced analysis. The Y chromosome of European bison was smaller than domestic cattle Y chromosome, and classified as acrocentric. Studies on the comparison of the Y chromosome structure of *Bison* and *Bos* species can bring very interesting results. Potentially identified differences within the Y-chromosome-linked sequences will allow to design a quick and easy-to-use test that unambiguously indicates father's descent. In 1999, a genetic test was developed based on the sequence of the 16S rRNA subunit, which made it possible to detect the presence of cattle-specific mitochondrial sequences in individuals of the *Bison* genus (Ward et al. 1999). Unexpectedly, when performing the analysis

Table 4. Number of NORs in the chromosome pairs of examined animals. The number of active NORs analyzed in metaphases is given in brackets.

	Numbers of NOR Chromosome					
	2 $\bar{x} \pm S$	3 $\bar{x} \pm S$	4 $\bar{x} \pm S$	11 $\bar{x} \pm S$	25 $\bar{x} \pm S$	28 $\bar{x} \pm S$
Niepołomice	1.22 ± 0.43 (81)	1.17 ± 0.52 (72)	1.17 ± 0.39 (42)	-	1.59 ± 0.51 (81)	1.75 ± 0.45(84)
Gołuchów	1.47 ± 0.51 (75)	1.36 ± 0.50 (57)	1.40 ± 0.51 (63)	-	1.44 ± 0.51 (69)	1.47 ± 0.52 (66)
Białowieża	1.50 ± 0.51 (81)	1.51 ± 0.52 (63)	1.38 ± 0.50 (66)	-	1.63 ± 0.50 (78)	1.75 ± 0.45 (63)
Muczne	1.39 ± 0.50 (75)	1.46 ± 0.52 (57)	1.22 ± 0.43 (66)	-	1.40 ± 0.51 (63)	1.54 ± 0.52 (60)
Bos taurus	1.20 ±0.41 (24)	1.36 ± 0.50 (19)	1.24 ± 0.44 (26)	1.05 ± 0.22 (22)	1.21 ± 0.42 (23)	-
Total	1.36 ± 0.47 (336)	1.37 ± 0.51 (268)	1.28 ± 0.10 (263)	1.05 ± 0.22 (22)	1.45 ± 0.49 (314)	1.63 ± 0.49 (273)

Table 5. Number of NORs in the cells of the examined animals.

	Cell		
	n	$\bar{x} \pm S$	min-max
Niepołomice	360	4.60 ± 1.76	1-8
Gołuchów	330	4.40 ± 1.76	1-8
Białowieża	351	4.60 ± 1.83	1-8
Białowieża	321	4.28 ± 1.59	1-7
Bos taurus	114	4.56 ± 1.66	2-8
Total	1476	4.49 ± 1.72	1-8

using this test in our laboratory, it turned out that over 75% of the studied European bison population showed the presence of sequences characteristic of the *Bos* genus (Nowak et al. 2008). Examination of selected sequences of the Y chromosome would allow the scanning of the European bison population and show the potential differences, not only between domestic cattle and European bison, but also between the separately maintained European bison lines: Lowland and Lowland-Caucasian.

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Contribution to the study of wild *Orchidaceae*, genus *Platanthera* L.C.M. Richard. Karyotype and C-banding analysis of two species from Italy

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Abstract. This study examined the chromosome numbers and karyotypes of two *taxa* of the genus *Platanthera* (*Orchidaceae*) from Italy. Cytological analyses showed 2n = 2x = 42 in *P. chlorantha* and *P. algeriensis*. Karyotype analysis revealed similarity between the species. The karyotypes are as follows: *P. chlorantha* consists of 34 metacentric + 8 submetacentric pairs and *P. algeriensis* consists of 36 metacentric + 6 submetacentric pairs. Both species possess a rather symmetrical karyotype. *P. chlorantha* has a very similar C-banding pattern to *P. algeriensis*. DAPI bright blocks were observed in *P. chlorantha*. These analyses also show the close relationship between the studied species.

Keywords: Chromosome number, C-Banding, heterochromatin content, karyotypes, *Orchidaceae*, *Platanthera algeriensis, P. chlorantha*.

INTRODUCTION

The genus *Platanthera* Rich., also known as "butterfly orchids", belongs to the subtribe *Orchidinae* (subfamily *Orchidoideae*) and consists of 100 to 200 species (Wood 2001; Delforge 2016; Efimov 2016 and references therein). The geographical distribution of *Platanthera* species covers most of the temperate areas of Europe, North Africa, Asia, New Guinea and North and Central America (Hulténand Fries 1986; Wood 2001; Efimov 2016), with 12 species widespread in Europe, six of which are found in Italy (Delforge 2016).

This genus is divided into 5 sections (Efimov, 2016), three of which are found in Europe: *P. hyperborea*, the most ancient clade, which has a Far-Eastern and North-American distribution, with only *P. hyperborea* (L.) Lindl. present in Iceland; the *P. oligantha* clade, with a circumpolar distribution, and finally the Eurasian section *Platanthera* (Delforge, 2016). *Platanthera* species are terrestrial, photosynthetic and – in very few cases – epiphytic or lithophytic. They are found in a variety of habitats, including meadows, temperate and boreal forests, bogs, fens, marshes and prairies. European *Pla-*

tanthera species are geophytes, characterised by a broad anther, 2 elongated root-tuberoids, a dorsal sepal and petals combining to form a helmet, a stigma without processes and an enlarged receptive surface with a nectariferous spur.

In cytological analyses performed on taxa of the genus *Platanthera*, the chromosome number was found to be 2n = 2x = 42 (Cauwet-Marc and Balayer 1986; Yokota 1987; Yang and Zhu 1988; Dalgaard 1989; D'Emerico 2001 and references therein). To date however, only four taxa exhibit polyploidy: *Platanthera hyperborea* (Dalgaard 1989) and *P. huronensis* (Nutt.) Lindl. (Sheviak and Bracht 1998), both with 2n = 4x = 84 chromosomes; *P. obtusata* (Banks ex Pursh) Lindl., which may be triploid in some populations with 2n = 63 (Tanaka and Kamemoto 1984); and the Nordic–Siberian *P. oligantha* Turcz. (= *P. obtusata* subsp. *oligantha*), which according to Webb (1980) is hexaploid (2*n* = 126).

As already mentioned above, six of these species are found in Italy: *Platanthera algeriensis* Batt. & Trab. 1892, *P. bifolia* subsp. *bifolia* (L.) Rich. 1817, *P. bifolia* subsp. *osca* Lorenz, Romolini, Romano & Soca 2015, *P. bifolia* subsp. *subalpine* Brügger, *P. chlorantha* (Custer) Rchb. and *P. kuenkelei* subsp. *kuenkelei* var. *sardoa* Lorenz, Akhalk, Baumann, Cortis, Cogoni & Scrugli 2012.

In this study, karyotype morphology and the distribution of heterochromatin in Italian specimens of *P. chlorantha* and *P. algeriensis* were studied for the first time. The aim of this study was to verify chromosome numbers and to compare the heterochromatin pattern of the above-mentioned species in order to verify similarities between them.

MATERIALS AND METHODS

The material studied in this investigation was gathered from natural populations of *Platanthera chlorantha* and *P. algeriensis* in Apulia and Sardinia (Table 1).

Mitotic chromosomes were prepared from immature ovaries, pre-treated with 0.3% colchicine at room temperature for 2h. For Feulgen staining they were fixed for 5 min in 5:1:1:1 (v/v) absolute ethanol, chloroform, glacial acetic acid and formalin, hydrolysed at 20 °C in 5.5 N HCl for 20 min (Battaglia 1957) and stained in freshly prepared Feulgen solution.

For C-banding, ovaries were fixed in 3:1 (v/v) ethanol–glacial acetic acid and stored in a deep-freeze for up to several months. Subsequently, they were squashed in 45% acetic acid; coverslips were removed by the dry ice method and the preparations were air-dried overnight. The slides were then immersed in 0.2N HCl at 60 °C for 3 min, thoroughly rinsed in distilled water and then treated with 4% Ba(OH)2 at 20°C for 4 min. After thorough rinsing they were incubated in 2xSSC at 60°C for 1h, and then stained in 3-4% Giemsa (BDH) at pH 7 (D'Emerico et al. 1996). For DAPI (4–6-diamidino-2-phenylindole) staining, ovaries were treated as for C-banding and stained using a buffered DAPI solution (0.6 mg/mL) for 5 min, followed by rinsing and mounting in glycerol buffer (1:1 v/v).

Chromosome pairs were identified and arranged on the basis of their length and any other evident karyomorphological feature. Heterochromatin content was assessed using MicroMeasure 3.3, a freeware program from Colorado State University (Reeves 2001). Karyotype symmetry indices – Mca (Mean Centromeric Asymmetry) and CVcl (Coefficient of Variation of Chromosome Length) – were used for the evaluation of karyotype asymmetry (Peruzzi et al. 2009).

The nomenclature used for describing karyotype composition followed Levan et al. (1964). A list of the examined specimens and their sampling locations is given in Table 1.

RESULTS AND DISCUSSION

Analysis of the somatic metaphases showed that the diploid chromosome number is 2n = 2x = 42 in both *Platanthera chlorantha* and *P. algeriensis*.

P. chlorantha, known as the "Greater Butterfly Orchid" (Lima-de-Faria 2020) was found to be diploid with 2*n* = 2*x* = 42 chromosomes (Fig. 1a), in agreement with previous reports (Scrugli 1980; Averyanov et al. 1985; Cauwet-Marc and Balayer 1986), with chromo-

Table 1. Taxon, sites, chromosome number, formula and percent heterochromatin in set of the chromosomes of species *Platanthera chloranta* and *P. algeriensis*. m, metacentric; sm, submetacentric.

Taxon	Site	Chromosome number (2n)	Formula	% Het in set
P. chlorantha	Martina Franca (TA) Balvano (PZ)	42	34m+8sm	25.50
P. algeriensis	Aritzo (NU)	42	34m+2m(sm)+6sm	22.43

Figure 1. Mitotic metaphase with Feulgen staining of *Platanthera chlorantha* (a) and *P. algeriensis* (b); 2n = 2x = 42. Bar = 5 μm.

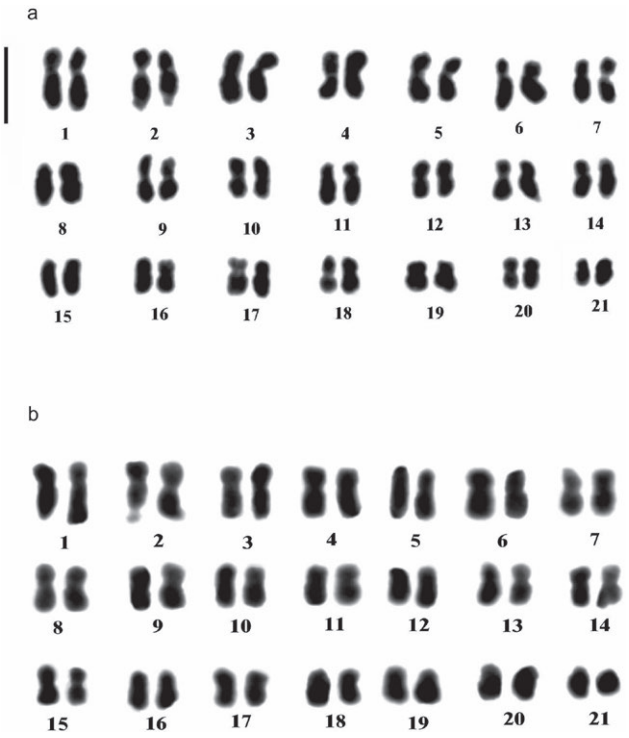

Figure 2. Diploid karyotypes of *Platanthera chlorantha* (a) and *P. algeriensis* (b). Bar = 5 μm.

somes that range in size from 4.04 to 1.8 μm at metaphase, and the arm length ratio was from 1.03 to 3.00. Six well spread-out metaphases were paired on the basis of chromosome size and centromere position and used for chromosome measurements. The karyotype consisted of 34 metacentric and 8 submetacentric chromosome pairs (Fig. 2a). The complement showed two chromosome pairs with secondary constrictions on the long arm (pair 2) and the short arm (pair 8).

This species possesses a fairly symmetrical karyotype (Mca = 18.50±1.09 and CVcl = 21.53±0.29), with metacentric chromosomes being the most frequent. It is interesting to note that this species has similarities in terms of dimensions and structure (such as the visibility of centromeres) with karyotypes of the *Anacamptis* group (2n = 2x = 36) (D'Emerico et al. 1996). Similarities with *Chamorchis alpina* (L.) Rich. (2n = 2x = 42) (D'Emerico and Grünanger 2001) and *Dactylorhiza romana* (Sebast.) Soò (2n = 2x = 40) (D'Emerico et al. 2002) can also be observed.

The C-banding analysis shows that constitutive heterochromatin was located in the centromeric regions of numerous chromosomes (Fig. 3a). One pair of chromosomes had the subtelomeric C-bands only on the short arm. Interphase nuclei exhibited a number of chromocentres equal to that of the constant bands (Fig. 3b). The centromeric regions of numerous chromosomes had bright fluorescence after staining with DAPI (Fig. 3c).

In *Platanthera algeriensis*, somatic cells showed 2n = 2x = 42 chromosomes (Fig. 1b). This species is similar to *P. chlorantha* apart from its greener flowers and its very different preferred habitat. In Europe this species is restricted to a few sites in Corsica, Sardinia, mainland Italy and Spain, but it also occurs in Algeria. In this species, similarities to the karyotype structure and C-banding of *P. chlorantha* were observed. Chromosome lengths were found to be between 3.90 and 2.18 μm. The karyotype consisted of 34 metacentric, 2 metacentric/submetacentric and 6 submetacentric chromosome pairs (Fig. 2b). In addition, this species possesses a symmetrical karyotype (Mca = 21.21±0.83 and CVcl = 18.19±0.60). Constitutive heterochromatin was also detected in the centromere regions of numerous chromosomes (Fig. 3d).

The present analysis of chromosome evolution showed that the species *P. chlorantha* and *P. algeriensis* are very close. Indeed, specimens of the two species in the present study exhibited practically the same karyotype and C-banding pattern. However, *P. algeriensis* seems to differ from *P. chlorantha* in that it has lower heterochromatin content (Fig. 3-d vs. Fig. 3-a). The small

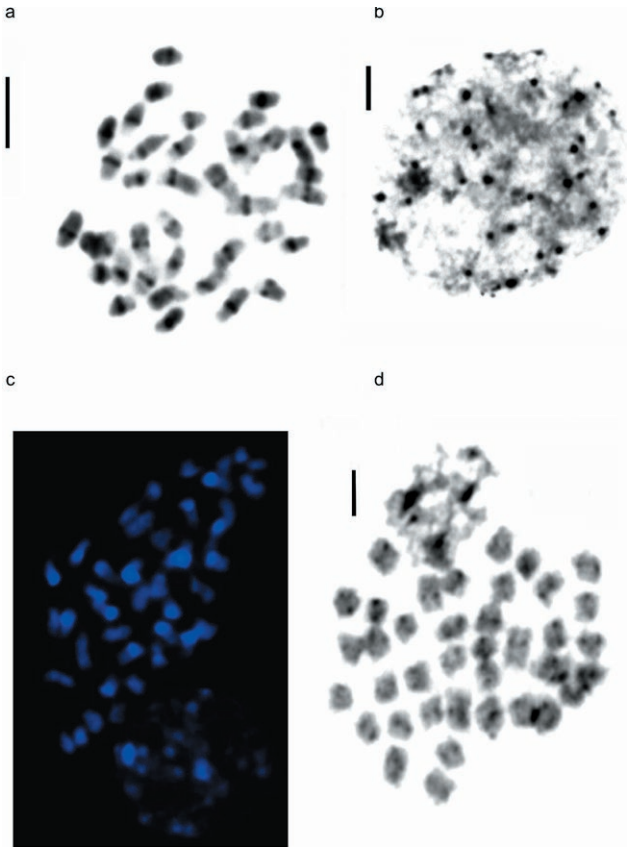

Figure 3. Giemsa C-banded mitotic metaphase of *Platanthera chlorantha* (a) and *P. algeriensis* (d); in *P. chlorantha*, interphase nuclei exhibit numerous chromocentres (b); *P. chlorantha* DAPI stained mitotic metaphase (c). Bar = 5 μm.

differences in the C-banding patterns found between the two species seem to indicate limited rearrangement of constitutive heterochromatin during their evolution.

In a study based on plastid DNA sequence variation, Pavarese et al. (2011) found that *Platanthera algeriensis* was characterized by haplotypes A and B, both of which are shared with *P. chlorantha*. In another study, Italian *Platanthera chlorantha* were found to form a single group with *P. algeriensis* from Tunisia and Sardinia (Bateman et al. 2012).

CONCLUDING REMARKS

The chromosome number 2n = 2x = 42 has been reported in 13 of the 17 genera for which data are available, although the subtribe *Orchidinae* includes about 50 genera (D'Emerico 2001; Felix and Guerra 2005). In this subtribe, the Internal Transcribed Spacer (ITS) phylogenies (Bateman et al. 2001; Bateman et al. 2003; Jin et al. 2017) show that the group *Pseudorchis-Amerorchis-Galearis-Platanthera* s.l. includes genera with the chromosome number 2n = 2x = 42 (Löve and Simon 1968; Löve 1981; Cauwet-Marc and Balayer 1986). Also it is suggested that *Dactylorhiza* s.l. and *Gymnadenia* s.l., which have 2n = 2x = 40, probably derive from 42 chromosomes (Pridgeon et al. 1997; Bateman et al. 2009).

As pointed out in the discussion, it is interesting to note the remarkable karyomorphological similarity in the species *Platanthera chlorantha*, *P. algeriensis* (this work), *Chamorchis alpina* (D'Emerico and Grunanger 2001), *Galearis diantha* (Schltr.) P. F. Hunt (Luo 2004), which have 2n = 2x = 42 chromosomes and *Dactylorhiza romana* (D'Emerico et al. 2002), which has 2n = 2x = 40 chromosomes.

Last but not least, in spite of the extensive cytogenetic literature that has built up over the years, little is known about the karyotype structure in other species of the genus *Platanthera* and related genera. Furthermore, to our knowledge, there are few studies of constitutive heterochromatin content, despite the fact that C-banding patterns provide extra information useful in assigning genomes.

We have a limited quantity of data on the *Platanthera* genus but it is possible to make some considerations on heterochromatin content. Indeed, *Platanthera chlorantha* and *P. algeriensis* show centromeric and subtelomeric heterochromatin, with a higher percentage in the former. On this basis, an evolutionary comparison is possible with the genera *Dactylorhiza* and *Gymnadenia*. Previous cytological studies using the traditional Giemsa C-banding technique have shown significant heterochromatin content in some species of these two genera (D'Emerico et al. 2002; D'Emerico and Grunanger 2001; Baumann et al. 2012). For example, in the genus *Gymnadenia*, *G. rhellicani* (Teppner and Klein) Teppner and Klein, *G. conopsea* and *G. odoratissima* (L.) Rich. have been found to possess numerous chromosomes with centromeric and telomeric heterochromatin. Similar banding patterns were previously observed in three species of the genus *Dactylorhiza*, including two diploids (*D. romana*, *D. saccifera* (Brogn.) Soò) and one polyploid (*D. urvilleana* subs. *phoenissa* B. Baumann and H. Baumann). Moreover, the distribution of C-banding patterns in *Dactylorhiza* karyotypes is of great cytological interest, although its nature can only be conjectured for the time being. Specifically, *D. romana* specimens have shown the chromosome numbers 2n = 40+1B and 2n = 40+2B, with one or two heterochromatic supernumerary chromosomes, which seems to suggest a possible evolutionary trend from 2n = 42 to 2n = 40 (D'Emerico et al. 2002).

For a better understanding of phylogenetic relationships within the genus *Platanthera*, cytogenetic analysis should be extended to other species. Moreover, new chromosome methods such as fluorescent *in situ* hybridization (FISH) and genomic *in situ* hybridization (GISH) will help to solve these problems using chromosomal analysis techniques.

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First cytogenetic register of an allopolyploid lineage of the genus *Aeschynomene* (Leguminosae, Papilionoideae) native to Mexico

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Abstract. A conventional cytogenetics analysis revealed for first time an allopolyploid lineage of the genus *Aeschynomene* in Mexico. The hybrid condition is confirmed after all the prometaphase and metaphase nuclei of the hybrids exhibited only one pair of SAT-chromosomes, confirming the existence of nucleolar dominance and amphiplasty. The karyotype formula for this lineage was $2n = 4x = 40 = 34$ m + 6 sm with a total diploid chromosome length (TDCL) = 28μm and an average chromosome size (AC) = 1.40 μm. Comparison of the karyotype and other chromosomal parameters with recent cytogenetics records for other species of the subgenus *Aeschynomene* included in the Nod-independent clade allows propose to *Aeschynomene evenia* and *A. scabra* as possible progenitors. Furthermore, other comparison of seedlings focused at the number of leaflets of the first four eophylls of the proposed parents and of the hybrid individuals allowed to observe coincidences that support the proposal made from the cytogenetic analysis. Evidence of "gigas" effects on flowers and fruits of hybrids is also shown.

Keywords: cryptic taxa, cytotype, karyotype, nucleolar dominance, SAT-chromosomes, secondary constrictions, seedlings.

I. INTRODUCTION

Aeschynomene Linnaeus (Leguminosae, Tribe *Dalbergieae* s. l.) is a diverse genus of subfamily Papilionoideae (Papilionoid legumes) distributed in the tropics and subtropics of the world (Lavin *et al.* 2001, Klitgaard and Lavin 2005). It comprises herbaceous and woody species, annual, repetitive and perennial with different ecological requirements. Several species contribute to supplement nitrogen to the soil through the production of nodular roots and stems in symbiosis with nitrogen fixing bacteria, so they are economically important as green manure (Alazar and Becker 1987; Fernandes 1996; Souza *et al.* 2012) and recently, *Aeschynomene evenia* C. Wright has been proposed as a model species in genetics to develop new agronomic

strategies in the engineering of nitrogen fixing nodules that enhance rice production (Arrighi *et al*. 2012, 2013). This taxon belongs to the group of 11 semi-aquatic species of *Aeschynomene* that have the property of being nodulated by photosynthetic *Bradyrhizobium* that lack the nodABC genes necessary for the synthesis of Nod factors and are grouped into the so-called Nod-independent clade (Chaintreuil *et al*. 2013; Brottier *et al*. 2018) and that correspond to the morphological series Indicae and Sensitivae (Rudd 1955).

The genus *Aeschynomene* traditionally included in the Aeschynomeneae tribe (Polhill *et al*.1981) and currently circumscribed in the Dalbergioid clade (Lavin *et al*. 2001; Wojciechowski *et al*. 2004) has evolved in different ecological niches and includes herbaceous forms, annual and perennial shrubs and trees up to 8 meters, with compound pinnate leaves and papilionoid flowers that are generally self-pollinated, although there is cross-pollination by bees (Rudd 1955; Fernandes 1996; Arrighi *et al*. 2014, Carleial *et al*. 2015). Other studies indicate that the genus *Aeschynomene* is not monophyletic and taxa with basifixed stipules and a campanulate calyx (subgenus *Ochopodium* Vogel) are more related to the genera *Machaerium* Persoon and *Dalbergia* Linnaeus f. than to taxa with medfixed stipules and a bilabiate calyx (subgenus *Aeschynomene* Léonard) (Ribeiro *et al*. 2007; Cardoso *et al*. 2012).

Currently *Aeschynomene* genus contains 170 (http://www.theplantlist.org) to 180 species (Klitgaard and Lavin 2005) 231taxa and cytotypes at four ploidy levels: diploid (2*x*), tetraploid (4*x*), hexaploid (6*x*) and octoploid (8*x*) (Index to Plant Chromosome Numbers; Kawakami 1930; Bielig 1997; Arrighi *et al*. 2012, 2014; Chaintreuil *et al*. 2016, 2018; Brottier *et al*. 2018). America, where most of the taxa are $2n = 20$ diploids, has been proposed as the center of origin of the genus, with a secondary distribution in Africa and Asia where polyploid species and some cases of aneuploidy predominate (Chaintreuil *et al*. 2018; Tapia-Pastrana *et al*. 2020).

Although it is clear that in the Dalbergioid clade, diploid $2n = 20$ genera predominate, with some polyploid and aneuploid species, in *Aeschynomene* there is currently a renewed interest in knowing to what extent polyploidy has contributed to the diversification and radiation of the group. In this respect Arrighi *et al*. (2014) revealed multiple hybridization/polyploidization events, highlighting the prominent role of allopolyploidy in the diversification of Nod-independent clade. In addition Chaintreuil *et al*. (2016) studied African *Aeschynomene* species and their data support the idea that the whole African group is fundamentally tetraploid and revealed the allopolyploid origin of *A. afraspera* J. Léonard ($2n = 8x = 76$) and *A. schimperi* Hochst. ex A.Rich. ($2n = 8x = 56$), where variations in the number of chromosomes also indicated possible dysploidy/aneuploidy events. In Mexico, *Aeschynomene* is represented by 31 species and infraspecific taxa including several endemisms. An investigation about the patterns of chromosomal evolution in Mexican species, including six taxa of the Nod-independent clade, showed the predominant of a basic $2n = 20$ diploid structure and evolutionary patterns related to the corresponding morphological series (Tapia-Pastrana *et al*. 2020).

In the present research, a conventional cytogenetic study was carried out to obtain the karyotype and analyze the level of ploidy in a Mexican population initially described as *Aeschynomene scabra* G. Don, where the size of the flowers, fruits and seeds generated suspicions about a possible hybrid origin. In addition as the sampled individuals exhibited floral morphotypes similar to those of *A. evenia* C. Wright and *A. scabra*, whose collection records in Mexico would support their participation in the hybridization process, the growth pattern of the first four eophylls was also compared in putative hybrids and their parental assumptions.

2. MATERIAL AND METHODS

2.1 Collection sites

Seeds of the putative hybrids were collected in the Municipio de la Huerta, Estado de Jalisco, Mexico, 19°29′N; 105°01′W (Carleial s/n, MEXU). The climate is semi-dry and warm. Mean temperature in the area is 25.2 °C, and there is a well-defined rainy season (average annual precipitation: 1107 mm) occurring from June to October (García-Oliva *et al*. 2002).

The seeds of *Aeschynomene evenia* and *A. scabra* were collected in the municipalities of Coyuca de Catalán (18° 19′ N; 100° 42′ W, JC Soto 15333 (MEXU)) and Arcelia (18°18′54′′N; 100°17′02′′W, JC Soto 15393 (MEXU)) respectively, in the State of Guerrero, Mexico. Both municipalities are part of the Tierra Caliente region. The predominant climate is warm subhumid with rains from June to September (average annual precipitation: 1100 to 1200 mm). The studied taxa are assigned to the infrageneric classification of Neotropical *Aeschynomene* sensu Rudd (1955) series Indicae of subgenus *Aeschynomene* and are part of the Nod-independent monophyletic clade (Chaintreuil *et al*. 2013), whose taxa are nodulated on roots and stems by photosynthetic *Bradyrhizobium* strains lacking the nod ABC genes necessary for the synthesis of Nod factors (Giraud *et al*. 2007).

2.2 Chromosome and karyotype procedures in putative hybrids

Seeds were collected in summer 2014 and from at least six plants. Batches of 40 seeds from each plant were used. The seeds were scarified and germinated in Petri dishes lined with a moist filter paper at room temperature and under natural light. Chromosomes at metaphase and prophase were obtained following the splash method (Tapia-Pastrana and Mercado-Ruaro 2001). All meristems were collected from 2-4 mm long roots pretreated with 2 mM 8-hydroxyquinolin for 5 h at room temperature and fixed in the fixative (ethanol: acetic acid=3:1). They were then treated with a mixture of 20% pectinase (Sigma) and 2% cellulase (Sigma) in 75 mM KCl for 60 min at 37 °C. After centrifugation at 1500 rpm for 10 min, the cell pellet was transferred to 75 mM KCl solution for 13 min at 37 °C. After two successive rinses with the KCl solution, they were again fixed in the fixative and subsequently rinsed twice more. One or two drops of the suspension of pellet were placed on clean slides, air-dried and stained in 10% Giemsa for 13 min. Preparations were made permanent using a synthetic resin.

At least ten metaphase plates of intact cells with well-spread chromosomes, no chromosome overlapping, and same contraction and ten prophase plates were photographed from each collection, using a microscope (Axioscope, Carl Zeiss) and analyzed for chromosome number determinations. Five photographs of metaphases with chromosomes having similar comparable degrees of contraction and centromeres clearly located were utilized to obtain the Total diploid chromosome length (TDCL), Total chromosome length (TCL), Average chromosome length (AC), the difference in length between the longest chromosome and the shortest chromosome (Range) and the longest/shortest chromosome ratio (L/S). The shapes of chromosomes were classified according to Levan *et al.* (1964) and the TF was obtained following Huziwara (1962). Furthermore, prometaphase cells were analyzed to verify both the number of nucleoli, and the behavior of the SAT chromosomes. The information thus obtained was compared with that recently recorded for *Aeschynomene evenia* and *A. scabra* in another cytogenetic study where the same method was used for karyotype analysis in *Aeschynomene* species and varieties (Tapia-Pastrana *et al.* 2020).

2.3 Seedlings and Eophylls

In order to compare seedling morphology in individuals of the supposedly hybrid population with those of *Aeschynomene evenia* and *A. scabra*, the development of 20 individuals grown in pots under greenhouse conditions was evaluated. Interest was particularly focused on the number of leaflets and the presence of hairs on their edges until the complete development of the fourth leaf. Eophylls at the first, second, third and fourth eophyllar nodes were referred to as E1, E2, E3 and E4, respectively following Schütz *et al.* (2019). Photographs of seedlings were taken with a Canon SX700 HS camera.

3. RESULTS

3.1 Karyotype analysis

A total of 410 cells were analyzed in metaphase and 16 in prometaphase and all exhibited a $2n = 4x = 40$ (Fig. 1 A-C). TDCL was 28 µm and AC 1.40 µm. The chromosomal range was 0.56 µm, the ratio 1.48 and a TF = 42.46. The karyotype formula was $2n = 4x =$ 34m + 6sm (Table 1). Consistently, in all prometaphase and metaphase nuclei, only one pair of submetacentric chromosomes was observed having lax secondary constrictions and macrosatellites in short arms (SAT-chromosomes) (Fig. 1 A-C). The karyotype exhibits small chromosomes (1.72-1.16 µm) clearly discernible, with predominance of metacentric chromosomes (m) and lacking subtelocentric chromosomes (st). This arrangement is consistent with a TF that describes a slightly asymmetric karyotype (Fig. 1D and Table 1). Occasionally the SAT-chromosomes were observed immersed in a single nucleolus.

3.2 Seedlings and Eophylls

The seedlings of the three taxa are illustrated in Fig. 2 A-C. Eophylls are stipulated, alternate, petiolate, pinnate, with alternate leaflets, have elliptic to oblong leaflets, a rounded apex, an entire margins, and one central primary vein in the three taxa under study. The leaflets did not present trichomes; both adaxial and abaxial surfaces are glabrous. The number of leaflets in the first four eophylls in seedlings of individuals of *Aeschynomene evenia*, *A. scabra* and putative hybrids are shown in Tables 2-4 respectively.

4. DISCUSSION

It is clear that the entire Dalbergioid clade (Adesmia, Dalbergia and Pterocarpus subclades) is dominated by $2n = 2x = 20$ species, with scattered polyploids and aneuploids (Lavin *et al.* 2001). In addition an ances-

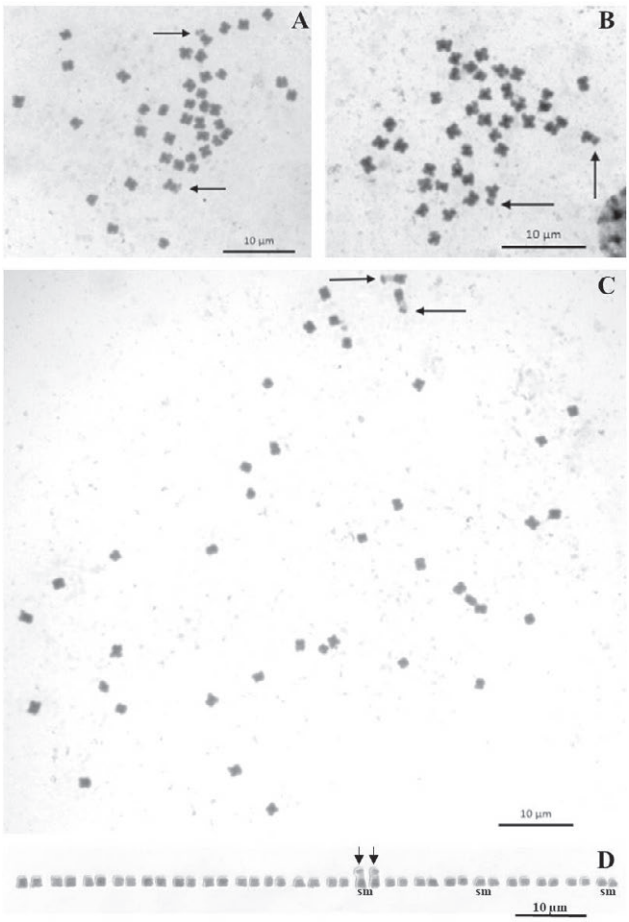

Figure 1. Mitotic metaphase cells of hybrid *Aeschynomene* 2*n* = 4*x* = 40. **A-C**, Metaphase chromosome plates in optimal spread; **D,** Karyotype 34m + 6sm. The chromosomes are aligned in decreasing order. Arrows point to secondary constrictions and satellites on short arms of submetacentric chromosomes.

Table 1. Average chromosome measurements obtained from five nuclei in metaphase of the hybrid population (2*n* = 4*x* = 40 = 34m + 6sm) under study.

CP	TCL (µm)	LLA (µm)	LSA (µm)	r	CT
01	1.72	0.96	0.77	1.24	m
02	1.63	0.89	0.73	1.21	m
03	1.59	0.89	0.69	1.28	m
04	1.55	0.81	0.72	1.12	m
05	1.53	0.81	0.70	1.15	m
06	1.50	0.82	0.66	1.24	m
07	1.48	0.83	0.63	1.31	m
08	1.46	0.79	0.66	1.19	m
09	1.44	0.79	0.63	1.25	m
10	1.42	0.79	0.61	1.29	m
11	1.38	0.74	0.64	1.17	m
12	1.36	0.89	0.46	1.93	sm*
13	1.34	0.78	0.55	1.41	m
14	1.31	0.70	0.59	1.18	m
15	1.28	0.72	0.55	1.30	m
16	1.24	0.83	0.40	2.07	sm
17	1.23	0.69	0.52	1.32	m
18	1.19	0.67	0.54	1.24	m
19	1.19	0.66	0.49	1.34	m
20	1.16	0.78	0.36	2.16	sm
TDCL	28.00				
AC	1.40				

Abbreviations: CP- chromosome pair; TCL- total chromosome length; LLA- length long arm; LSA- length short arm; r- arm ratio; CT-chromosome type; TDCL- Total diploid chromosome length; AC- Average chromosome length; m- metacentric; sm- submetacentric; *- satellite. Abbreviations: CP- chromosome pair; TCL- total chromosome length; LLA- length long arm; LSA- length short arm; r- arm ratio; CT-chromosome type; TDCL- Total diploid chromosome length; AC- Average chromosome length; m- metacentric; sm- submetacentric; *- satellite.

tral state reconstruction performed in a phylogeny based on *ITS* + *matK* of the *Aeschynomene* genus and related genera indicated that diploidy is the ancestral condition in the entire group reviewed (Brottier *et al*. 2018). However, the role of allopolyploid speciation events in the origin of new taxa is now recognized (Arrighi *et al*. 2014).

As far as we know, the first assumption about of hybridization in *Aeschynomene* is attributed to Rudd (1955) who pointed out that the species with the widest distribution within the Indicae series (Nod-independent clade) tend to be more variable and intergrade with their neighbors. Later, Verdcourt (1971) suggested that specimens of *Aeschynomene rudis* Bentham (also into Nod-independent clade) with large flowers could be of polyploid origin, without pointing out the possible duplication mechanism involved, auto or allopolyploidy. To date, several studies have shown that the clade of *A. evenia* is mainly diploid (2*n* = 2*x* = 20), however some species such as *A. indica* Linnaeus (2*n* = 4*x* = 40, 2*n* = 6*x* = 60) seem to be of recent allopolyploid origin (Arrighi *et al*. 2014; Chaintreuil *et al*. 2018; Tapia-Pastrana *et al*. 2020). Furthermore, it has been found that all species of the group *A. afraspera* are polyploid (2*n* = 4*x* = 28, 38, 40; 2*n* = 8*x* = 56, 76) and have a common AB genomic structure (Chaintreuil *et al*. 2016). In facts phylogenetic relationships between diploids and polyploids elucidated from *ITS* sequences show that in the Nod-independent clade, species such as *A. evenia*, *A. scabra* and *A. rudis* participate in the hybridization/polyploidization events and formation of polyploid complexes that have contributed to the radiation of this group (Arrighi *et al*. 2014).

Figure 2. Seedling morphology of *Aeschynomene* under study until the complete development of the fourth eophyll **A**, *Aeschynomene evenia*; **B**, *A. scabra*; **C,** hybrid of *Aeschynomene*.

Table 2. Number of leaflets up to the fourth eophyll in *Aeschynomene evenia*.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
E1	10	10	10	10	10	10	10	10	10	10	10	9	10	9	8	10	9	10	10	8
E2	12	12	12	12	11	12	14	12	10	14	12	12	14	12	10	13	12	11	10	11
E3	15	16	15	14	14	16	17	15	14	16	16	15	17	12	12	16	16	12	14	12
E4	16	18	16	16	16	16	18	18	14	16	18	16	18	16	15	16	16	14	16	15

Table 3. Number of leaflets up to fourth eophyll in *Aeschynomene scabra*.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
E1	10	8	10	10	10	10	10	8	10	9	10	10	10	10	10	8	8	8	8	10
E2	12	15	14	14	14	14	14	12	16	13	14	13	14	14	14	12	12	13	15	14
E3	20	20	22	18	18	18	19	19	20	17	17	18	18	16	18	19	19	19	20	16
E4	24	25	27	23	22	22	22	22	24	22	22	22	22	21	22	22	22	20	25	21

Table 4. Number of leaflets up to fourth eophyll in hybrids.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
E1	10	10	8	10	10	9	10	8	8	10	8	10	8	10	10	8	10	10	8	10
E2	14	14	12	14	13	14	14	14	14	14	14	14	14	16	14	14	14	14	14	10
E3	18	20	18	20	20	19	20	20	20	19	18	20	18	20	20	20	20	15	18	14
E4	21	25	22	23	23	26	23	24	23	24	23	26	22	27	22	22	23	22	23	14

In the present investigation, the chromosomal number obtained in all the nuclei analyzed from the individuals under study was $2n = 4x = 40$, which undoubtedly shows that they are polyploid cells and that the individuals from which they come integrate a polyploid lineage not previously detected in Mexico (Rudd 1955; Tapia-Pastrana *et al*. 2020). The origin of the polyploidy (auto or allopolyploidy) were established easily from the number of SAT chromosomes unambiguously identified both in nuclei in prometaphase and metaphase and by their position in relation to the nucleolus.

Indeed, polyploidy, the process of genome doubling that gives rise to organism with multiple sets of chromosomes, is recognized as an important process in plant evolution, a major mechanism of adaptation and is often invoked as a driver of diversification (Ramsey and

Schemske 1998; Soltis *et al*. 2009) and it is likely to be one of the most predominant mechanisms of sympatric speciation in plants (Otto and Whitton 2000). It can act alone, resulting in autopolyploidy, or in concert with hybridization, producing allopolyploids, and both modes lead to plant speciation. It should be mentioned that in the process of polyploidization by total gene duplication (autopolyploidy) the number of satellites present in a diploid species is also doubled, since this does not involve loss or suppression of the nucleolar function, the NOR regions associated with secondary constrictions in SAT-chromosomes are they show lax and therefore satellites are clearly appreciated. It is known that NORs contain tandemly arranged highly reiterated ribosomal rRNA genes coding for 18S-5.8S-26S rRNA whose expression is under epigenetic control (Pikaard 2000). For example, *Medicago sativa* Linnaeus, a recognized autotetraploid exhibits four macrosatélites in metaphase cells (Falistocco 1987). In contrast, plants of allopolyploid origin as cotton (*Gossypium hirsutum* Linnaeus $2n = 4x = 52$ AADD, Endrizzi *et al*. 1985), wheat (*Triticum aestivum* Linnaeus $2n = 6x = 42$, AABBDD, Lacadena and Cermeño 1985; Friebre *et al*. 1995) and canola (*Brassica napus* Linnaeus $2n = 4x = 38$ AACC; Xiong and Pires 2011) undergo inactivation of the regions of the nucleolar organizer (NOR) of one of the parental genomes, silenced by the effect of nucleolar dominance (Navashin 1934) and consequently a smaller number of satellites is recorded (Doyle *et al*. 2008; Ge *et al*. 2013). It is, rDNA loci may be additive in number, but then exhibit differences in gene expression. Interspecific hybrids often have rRNA genes of one parent functionally dominant over the rRNA of the other parent, and there are many examples of such regulation of rRNA gene activity in allopolyploids (Pikaard 2000; Pires *et al*. 2004). Comparative analyses of nucleolar organizer regions (NORs) of somatic metaphase chromosomes made by phase contrast, C-banding and silver staining have demonstrated that the activity of the NORs of certain chromosomes can be suppressed or partially inhibited by the presence of other SAT-chromosomes.

The NOR competition is cytologically expressed as amphiplasty: a term proposed to denote morphological changes which occur in chromosomes following interspecific hybridization (Rieger *et al*. 1976). The secondary constriction of the SAT-chromosome of one of the parental species is missing in the hybrid and the satellite is retracted onto the chromosome arm as a consequence (Lacadena and Cermeño 1985). Thus, in the *Hordeum murinum* Linnaeus complex (Poaceae, Triticeae), tetraploid and hexaploid cytotypes arising from hybridization exhibit only a pair of chromosomes with secondary and satellite constrictions (Cuadrado *et al*. 2013). In fact, the inactivation or epigenetic silencing of ribosomal genes is one of the most common phenomena in hybrid and polyploid members of Triticeae Linnaeus (Cermeño and Lacadena 1985; Carmona *et al*. 2016) and one of the first examples of differential gene expression discovered in plant hybrids nearly a century ago (Navashin 1934; Matyášěk *et al*. 2007). In the present work, the repressive effects on NORs from allopolyploid population are cytologically expressed (amphiplasty) as the suppression of a secondary constriction clearly observed in all their complements (Fig. 1 A-D).

The karyotype exhibited in hybrid individuals (34m + 6sm) (Fig. 1D and Table 1) coincides in several respects with that expected at a cross between *A. evenia* ($2n = 2x = 7$m + 3sm) and *A. scabra* ($2n = 2x = 10$ m) (Fig. 2 in Tapia-Pastrana *et al*. 2020). For example, the number of sm chromosomes in *A. evenia* agrees with the 6sm in hybrid individuals. In addition to submetacentric chromosomes, these individuals exhibit metacentric chromosomes whose predominance is consistent with the karyotype formulas described in their putative relatives, whose complements lack subtelocentric chromosomes (Tapia-Pastrana *et al*. 2020). There is a coincidence between THC and AC and even the morphology of the SAT-chromosomes (submetacentrics with macrosatellites in short arms) and their position in the karyotype is very similar to that recently described in *A. scabra* (Tapia-Pastrana *et al*. 2020). Therefore we propose to *A. evenia* and *A. scabra* as progenitors of the allopolyploid population ($2n = 4x = 40 = 34$m + 6sm) registered in this work. The reasoning is simple: if a diploid species is involved in the origin of a tetraploid cytotype, its chromosomes must be present in it. The same is true if tetraploid forms are involved in the origin of hexaploid forms (Cuadrado *et al*. 2013). In Mexico, recent collection data shows that populations of both species occupy overlapping ranges in some central areas of the country where *A. evenia* is considered an introduced species (Arrighi *et al*. 2013; Chaintreuil *et al*. 2018; Tapia-Pastrana *et al*. 2020).

This new proposal is not surprising, since previously the Indicae series species grouped within Nod-independent clade, including *A. evenia* and *A. scabra*, have been identified as progenitors in allopolyploids and in the formation of polyploid complexes, although attempts at hybridization have failed to form fertile individuals (Arrighi *et al*. 2014). Regarding the identity of the allopolyploid taxon recorded here, it can be argued that a detailed review of its complete morphological characters (data not shown) suggests that it shares characteristics described for *Aeschynomene rudis* particularly in the shape and size of flowers, fruits (hispidulous, verru-

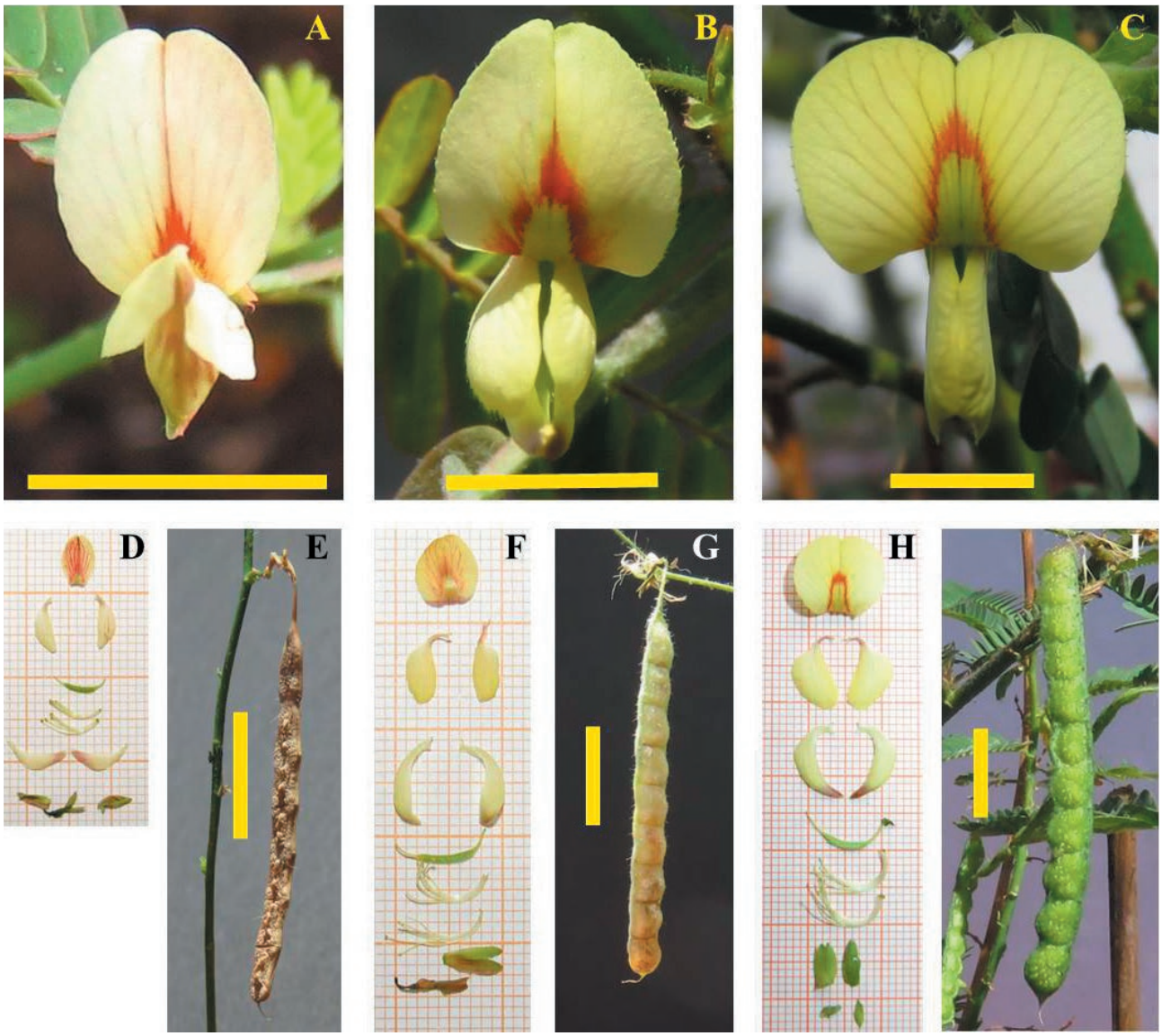

Figure 3. Floral morphotypes (above), dissected flowers and fruits (below) of the taxa under study. **A**, **D** and **E**, *Aeschynomene evenia*; **B**, **F** and **G**, *A. scabra*; **C**, **H** and **I**, hybrid of *Aeschynomene*. All three taxa exhibit typical pea or papilionoid flowers. These zygomorphic flowers comprise a standard (vexillum or banner) petal (adaxially placed), two lateral petals (wings) and two (usually partially fused and abaxially placed) keel petals, which conceal the androecium and gynoecium. The fruits have similar characteristics and are mainly differentiated by their size. Above scale bar = 0.5 cm, below = 1.0 cm.

coses, or muricate at the center) and seeds (Rudd 1955). However, it also recalls the robust version of *A. scabra* described by Rudd (1955). The existence of cryptic taxa in *Aeschynomene* as well as the need for broader sampling to detect new cytotypes has already been pointed out (Brottier *et al.* 2018, Chaintreuil *et al.* 2018) and the results of this study confirm this.

Regarding the results obtained from the seedling comparison, these seem to support a close relationship between the individuals of the three populations studied (Fig. 2, Tables 2-4). In principle, the observed intervals in the number of leaflets per eophyll (E1-E4) show some uniformity, particularly E1, whose interval (8-10 leaflets) was repeated in the three populations. In intermediate eophylls (E2-E4) a close concordance is observed between *A. scabra* and the hybrid population, while in *A. evenia* the number of leaflets was lower in correspondence with the taxonomic description of this species (Rudd 1955). Furthermore, the morphology of the eophylls was similar and in all populations the leaflets

exhibited entire margins, without trichomes and with a central primary vein.

Polyploids are known to often have novel phenotypes that are not present in their diploid progenitors or that exceed the range of parent species ("gigas" effects) (Ramsey and Schemske 2002; Ramsey and Ramsey 2014). In this sense, Fig. 3 shows floral morphotypes, dissected flowers and fruits of the populations studied here, where similarities are observed, but the differences in size of such characters are highlighted. The results obtained in this study confirm that in the Nod-independent lineage within the genus *Aeschynomene*, hybridization and polyploidization play a relevant role in the formation of species and those taxa such as the polymorphics *A. evenia* and *A. scabra* actively participate in it.

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Possible hybrid speciation for two Malagasy species of *Piper* L. (*Piperaceae*)

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Abstract. Two new species of genus *Piper* L. from Madagascar: *Piper malgassicum* Papini, Palchetti, M. Gori & Rota Nodari and *Piper tsarasotrae* Papini, Palchetti, M. Gori & Rota Nodari, were analyzed to investigate their phylogenetic position and evolutionary history. Both plastidial and nuclear markers were used for sequencing. The plastidial markers (*ndh*F and *trn*L intron) showed a close relationship between the two species with respect to the other species of *Piper*. Both species appeared phylogenetically related to the African *P. guineense* and the Malagasyan/Mascarenhas endemic *P. borbonense*. The nuclear marker (*G3pdh*) amplification produced two separate sets of sequences: "long" sequences and "short" sequences, characterized by some long deletions. Analyzing together the nuclear sequences, we observed that the "long" sequence of *P. tsarasotrae* had a stricter relationship to the African accessions of *P. guineense*, while the accession of *P. malgassicum* was more strictly related to *P. borbonense*. On the contrary both "short" sequences of *P. malgassicum* and *P. tsaratsotrae* resulted phylogenetically related to Asian accessions and more distantly related to the formerly cited species. This unexpected result was tentatively explained with a more ancient hybridization event between an ancestor of *P. malgassicum* and *P. tsarasotrae* (and possibly *P. borbonense*) and an Asian species of *Piper*. The Asian contribution would have produced the ancestors carrying the "short" sequences. A more recent hybridization event would have led to the separation of *P. malgassicum* from *P. tsarasotrae* with an African pollen-derived genome contribution from *P. guineense* or, more probably, an ancestor thereof, to an ancestor of *P. tsarasotrae*. The chromosome numbers of *P. tsarasotrae* (2n = about 38) and *P. malgassicum* (2n = about 46), were more similar to the Asian species than to the American species. Unfortunately, no chromosome number of the African species *P. guineense* is currently available, to compare the chromosomal numbers.

Keywords: *Piper malgassicum*, *Piper tsarasotrae*, *Piperaceae*, chromosomes, hybridization, DNA sequences, *G3pdh*, *trn*L, *ndh*F, Malagasy biodiversity.

1. INTRODUCTION

Genus *Piper* L. (Piperaceae) is one of the largest genera of Angiosperms, with more than 2000 species (Quijano-Abril et al. 2006) and were considered belonging to a basal group of angiosperms, the so called "paleoherbs" (Loconte et al. 1991).

Piper is a pantropical genus developing highly variable growth forms (Isnard et al. 2012), with the highest biodiversity in the American continent with a number of species ranging from 500 (Burger 1972; Tebbs 1993), to 1100 (Jaramillo and Manos 2001), later increased to more than 1800 (Ulloa Ulloa et al. 2017), many of them with a small distribution area (Quijano-Abril et al. 2006).

The separation of species is often tricky, due to the small size of the floral parts and hence the number of synonyms may be high (Suwanphakdee et al. 2016), while other species tend to get naturalized (Smith et al. 2008). While only two species are known as native of the African continent, *P. guineense* Thonn. and *P. capense* L. f., more species are known of Madagascar, even if some of them are known only for a single or few herbarium samples. The currently recognized species in Madagascar are *P. heimii* C. DC, *P. pachyphyllum* Baker and possibly *P. borbonense* (Miq.) C. DC., described for the Bourbon island, nowadays La Reunion (Weil et al. 2017), belonging to the Mascarenhas Islands. However, its presence in Madagascar was affirmed by De Candolle (1869; 1923). The fact that *P. borbonense* is cultivated makes more complex to understand its real distribution area (Palchetti et al. 2018).

Piper malgassicum Papini, Palchetti, M. Gori, Rota Nodari and *Piper tsarasotrae* Papini, Palchetti, M. Gori, Rota Nodari, were recently described as new Malagasy species (Palchetti et al. 2018) and are of economic interest, since their dried fruits are often mixed with *P. borbonense* to produce the typical Malagasy spice called in local language "voatsiperifery" pepper.

The aim of the investigation was to understand how the malagasyan species might have been originated and their relationships with the African and the Asian species. This knowledge will help to understand how the Malagasyan species used as spices may be related to *P. nigrum* with possible future biotechnological implications. The chosen method for anwering the research goal was the analysis of DNA sequences both of nuclear and plastidial origin and the chromosome numbers of *P. malgassicum* and *P. tsarasotrae*.

2. MATERIALS AND METHODS

A first round of sample collection within the internal area of Madagascar was conducted in 2016 and the samples have been submitted to analyses. The results have been reported in Palchetti et al. (2018) but, in order to get a deeper knowledge about the genetic asset of the two species and to confirm the obtained results e second round of sample collection has been carried on in 2019. 4 new plants were collected in two different areas of the Ambositra region in Madagascar. The first 2 plants, belonging to the *P. malgassicum* type, were collected in the tropical rainy forest of Vohiday and the second 2 plants, belonging to the *P. tsarasotrae* type, in the semi-dry area of the Tsaratsotra village. These plants were compared with the samples of *P. tsarasotrae* and *P. malgassicum* which have been used for a previous investigation that included the description of the species (Palchetti et al. 2018). Samples were conserved either in ethanol 96% either as herbarium sample by the ET (Tropical Herbarium of Florence, CSET, https://www.bio.unifi.it). Some seeds were also germinated in Florence for karyotyping. The DNA used for this work was extracted from tissue conserved in ethanol 96% (Murray et al. 1996; Bressan et al. 2014).

DNA was extracted from 40 mg of the ethanol preserved leaves after drying under vacuum. The starting material was inserted in 2 ml tube, together with tungsten carbide beads, frozen in liquid nitrogen and finely ground in a tissue homogenizer (Tissue Lyser ®, Qiagen). DNA was extracted using Invisorb Spin Plant Mini kit (Stratec molecular®) according to the manifacture's guidelines.

Amplification of the trnL (UAA) intron (*trn*L) and the low copy nuclear gene glyceraldehyde 3-phosphate dehydrogenase (*G3pdh*) followed respectively the protocols by Taberlet et al. (1991) and Strand et al. (1997).

Two new primer pairs were designed using the chloroplast genome sequence of *P. kadsura* (GenBank®: KT223569.1) as template to cover the entire NADH dehydrogenase F (*ndh*F) plastid gene: ndhF-F3_forward 5'-AGGTTCTTATCGAGCCGCTT-3' and ndhF-F3_reverse 5'-GTAAGAAGAAATGCGCCCCC-3' and ndhF-F10_forward 5'-CTTCGCCGTATGTGGGCTTT-3' and ndhF-F10_reverse 5'-TCGACCAAAAGCAAGCAAGAG-3'. The amplicons have been directly and bi-directionally sequenced by using the corresponding primers for each amplified sequence. Since direct sequencing of *G3pdh* showed fragments of extra peaked sequencing data, we proceeded with cloning with InsTAclone PCR Cloning Kit (Thermo Scientific®) of the *G3pdh* amplification products. Several colonies for each cloned sample were amplified using T7 and SP6 primers whose sites are located at the boundaries of the cloning region. PCR products were purified using the QIAquick PCR Purification Kit (Quiagen) and sent to the University of

Figure 1. Alignment of the long and short *G3pdh* sequences isolated from *P .malgassicum* and *P. tsarasotrae* using BioEdit software (Hall 1999). Shaded fragments represent the primers used for amplification.

Florence internal sequencing service CIBIACI (www.cibiaci.unifi.it). Manual correction and assembly of the sequences was performed using the software Multalin (Corpet 1988) and MEGA7 (Kumar et al. 2016). Unexpectedly, two DNA sequences were obtained, after removing the cloning vector fragments, showing a different size: 965bp and 1058bp which were named "short" and "long" sequences respectively (Figure 1).

At a first sight only the long sequences of *G3pdh* have been considered as the right ones because, as

observed by Smith et al. (2008), no paralogs have been detected in a great deal of other Piper species and therefore other results have been discarded as PCR artifacts. In the present work a second thorough revision of the sequencing output has been carried on and showed the presence of overlapping peaks in all the samples and an additional round of analysis of the colonies confirmed the presence of the short sequences. To rule out any doubt, two additional specimen for each species has been collected and submitted to amplification and cloning in order to confirm the presence of the short sequence. As all the samples showed the same pattern, we decide to use also this "short" sequence to study the phylogeny of these two piper species, by comparing with all the accession present online.

The sequences used during our investigation are available in GenBank®: *Piper tsarasotrae G3pdh* long sequence (MH234634), *G3pdh* short sequence (MT793801), *trn*L (MH234638), *ndh*F (MH234636) and *Piper malgassicum*: *G3pdh* long sequence (MH234633), *G3pdh* short sequence (MT793800), *trn*L (MH234637), *ndh*F (MH234635).

2.1. Phylogenetic analysis

The DNA sequences were aligned with CLUSTALX 2.0 and checked by eye for manual adjustment. The plastidial and the nuclear sequences were aligned separately to produce matrices that were later combined with the software combinex2_0.py (Python version 2.6.4; Biopython 1.57), by A. Papini, released under GPL license and available at www.unifi.it/caryologia/PapiniPrograms.html as implemented in Bandara et al. (2013) and in Simeone et al. (2016).

The phylogenetic analysis was executed on both cpDNA (*ndh*F and *trn*L) and nuclear sequences (*G3pdh*). Maximum parsimony analysis was performed with PAUP* 4.0b1 (Swofford 1998, 2001). The genbank sequences of *P. humistratum* Görts & K. U. were used as outgroups both in the nuclear and the plastid genes matrix, following the previous phylogenetic analysis by Smith et al. (2008). This sequences used as outgroup resulted belonging to the sister clade with respect to the clade containing the African species and the other related clades in Smith et al. (2008). References of the other species used in the analysis are summarized (with GenBank® codes) in Table 1 in Smith et al. (2008). All characters had equal weight and unordered state transitions. Gaps were coded with the "simple indel coding" model (Simmons and Ochoterena 2000), with the software Gapcoder (Young and Healy 2003) and added to the final matrix after the DNA sequences as in Papini et al. (2004).

The evolutionary model implemented in Mrbayes for treating gaps was the same as that proposed by Lewis et al. (2001) for treating morphological data, the Mk model, justified as simple absence/presence of the character without *a priori* assignment of different weights.

We used MrMODELTEST 2.0 (Nylander 2004) to choose the best evolutionary model of DNA sequences on the basis of the Akaike information criterion (Akaike 1974). The best model was used as settings with MrBayes 3.2.7 (Ronquist et al. 2012) for Bayesian Inference. A maximum likelihood (ML) phylogenetic analysis was carried out with RaxML (Stamatakis 2014) and the resulting trees were edited with Figtree (Rambaut and Drummond 2010). We mapped the support on the tree branches with the results of the Bayesian phylogenetic analysis after removing the first trees with low likelihood values as "burn-in", as in Papini et al. (Papini et al. 2007; Papini et al. 2011). The remaining trees were used to produce a 50% majority-rule consensus tree in which the percentage indicated on branches was used as a measure of the Bayesian posterior probability.

2.2. Karyological analysis

Chromosomes images were obtained from somatic mitoses recorded from root tips of only one plant living in a pot. The procedure was the same as in Mosti et al. (2011) and Mousavi et al. (2013), with a pretreatment in 8-hydroxyquinoline and fixation in Carnoy. Then the material was hydrolyzed in HCl and then stained with Lacto-propionic-orceine.

We observed metaphase plates of meristematic cells, with the technique of fresh squashes of root tips. Chromosome counts were made during direct observations with the microscope, and later recounted on enlarged digital images. Images were recorded with a microscope Leica DM RB Fluo.

3. RESULTS

Amplification of two plastid fragments named *ndh*F and *trn*L intron was carried on and the amplicons correctly sequenced producing reads of 1860 bp and 920 bp, respectively. Cloning of the amplicon of the nuclear gene *G3pdh* of *P. malgassicum* and *P. tsarasotrae* allowed to isolate two haplotypes, which were named "long" (1060bp for *P. malgassicum* and 1045bp for *P. tsarasotrae*) and "short" 965 bp (for both species) after their size. We used a total of 71 sequences, considering separately the short and long sequences of *P. malgassicum* and *P. tsarasotrae* for *G3pdh* and the plastid sequences

Figure 2. Maximum likelihood tree produced by RAXML with nuclear sequences. The supports above or below the branches are, respectively, the bootstrap resampling support with maximum likelihood criterion produced by RAXML, and the bayesian support calculated including the information derived from indels. In case the bayesian support is lower than 50, it is not indicated on the figure.

matrix. The total alignment of the *G3pdh* region was 1127 nucleotides long including gaps. The final parts of the sequences were very variable and hence the alignment was ambiguous. For this reason, we excluded the characters from position 957 to 1127. The rest of the alignment was used for indels (gap) coding (with the

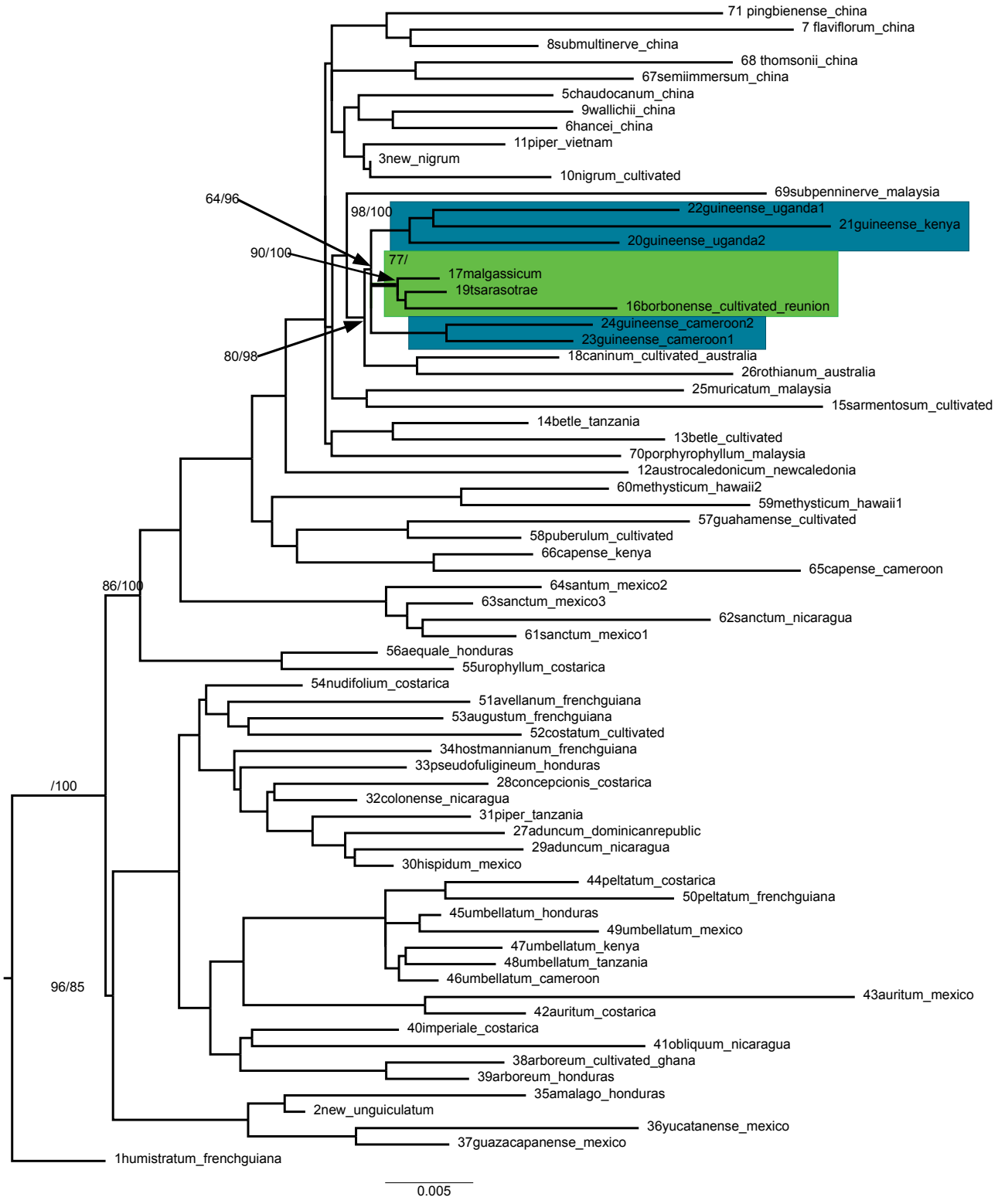

Figure 3. Maximum likelihood tree produced by RAXML with chloroplast sequences. The support indexes indicated on the tree are the same as in Figure 2 (maximum likelihood bootstrap and bayesian support).

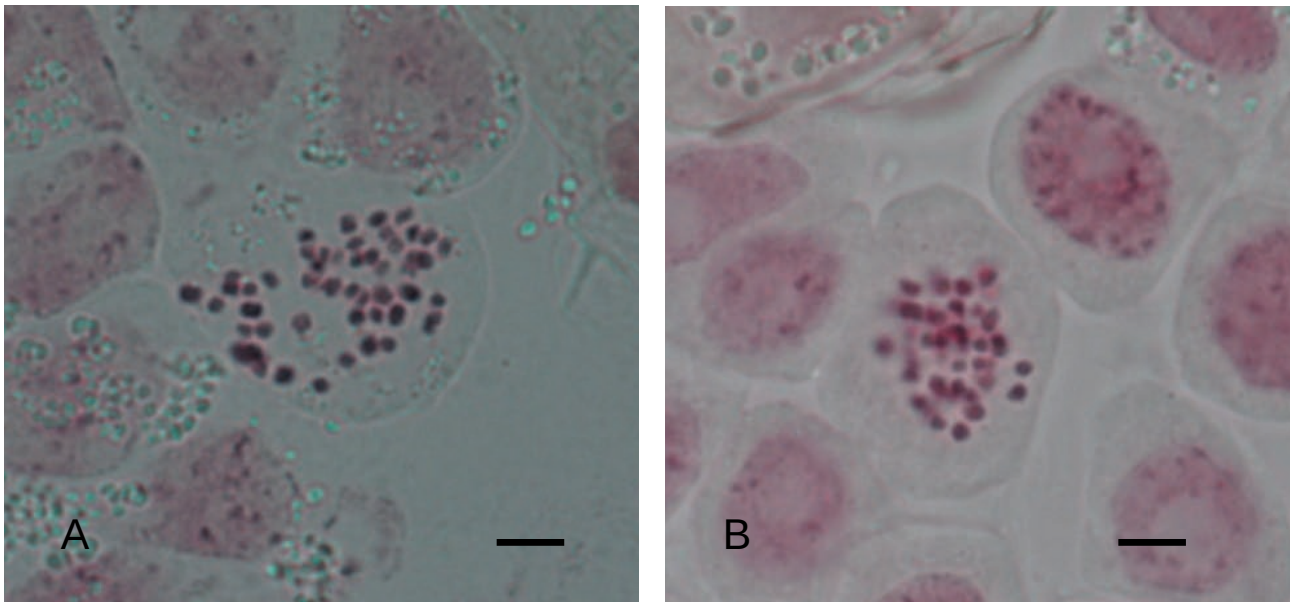

Figure 4. Chromosomes. A) *P. malgassicum* number of chromosomes: 2n = about 46. Bar = 5 μm; B) *P. tsarasotrae*: 2n = about 38. Bar = 5 μm.

software gapcoder), resulting in further 99 characters that were inserted after the nucleotide sequences. The plastid genes *ndh*F and *trn*L were inserted one after the other in the sequence, producing an aligned matrix of 2016 characters. The coding of indels resulted in further 115 characters. RAxML applied on the nuclear *G3pdh* matrix (indels coding excluded) produced a maximum likelihood tree with bootstrap support obtained with 1000 replicates (Figure 2).

The support on branches corresponds to maximum likelihood bootstrap support (left) and Bayesian support with gaps (on the right). The same method was using for the plastid matrix (Figure 3).

Comparing the two maximum likelihood trees, the one based on nuclear DNA data (*G3pdh* sequences) and that obtained with plastid markers, we could observe that in the first case *P. malgassicum*, clustered together and as sister group of *P. borbonense* (Figure 2), another species from an island, La Reunion, which lies relatively close to Madagascar. This relationship is corroborated by 100% maximum likelihood bootstrap (MLS) and bayesian (BS) support. The other Malagasy species, *P. tsarasotrae*, typical of arid forest, was more strictly related to the entries of the African species *P. guineense*, with 100% MLS and 100% BS. All these species formed a well characterized clade with 89% MLS and 100% BS and their closest species appeared to be Asian species *P. caninum*, (Figure 2). The BS without considering gaps coding gave the same support in this clade.

The "short" sequences of *G3pdh* of both *P. tsarasotrae* and *P. malgassicum* clustered together within a group of Asian species, mainly originating from Malaysia and Australia with 98% MLS and 100% BS (Figure 2).

The (phylogenetic) story told by the data obtained from chloroplast genome sequences was quite different: the Malagasyan species *P. tsarasotrae* and *P. malgassicum* clustered together with the phytogeopraphically close *P. borbonense* with 90% MLS and 100% BS, while the 5 accessions of the African *P. guineense* were in a more external condition with respect to the former group and separated in two groups, one from Cameroon (NW Africa) and one from Uganda/Kenya (Central-East Africa). All these species together formed a monophyletic group with 64% MLS and 96% BS (95% bayesian support in the analysis without gaps). Also in this case *P. caninum*, together with *P. rothianum*, was the outgroup to the African + Malagasy species (Figure 3) with 80% MLS and 98% BS (99% without gaps). Adding indels data to the matrix did not appear to increase the support value of nodes in the plastidial genes tree.

The counted chromosome numbers varied from 2n=46±2 in *P. malgassicum* (Figure 4A) to 2n=36± 2 in *P. tsarasotrae* (Figure 4B). The uncertainty in the counts, that should be taken only as preliminary result, derived from the small size of the chromosomes (many of them less than 1 μm of length), the low amount of metaphases in the root tips of the plants cultivated in Florence and the apparently small size of the mitotic spindle, leading

to partial overlapping of many of the small chromosomes.

The two currently known areal of the two species (two new localities discovered here) is shown in Figure 5.

4. DISCUSSION

The fact that the phylogenetic history based on the chloroplast markers told a different tale with respect to the tree produced with nuclear markers may be explained with a possible ancient hybridization/introgression event with pollen coming from an ancestor of the African *P. guineense* and reaching the ancestor of *P. tsarasotrae*, that would hence share some part of the nuclear genome with the African species. The only species of *Piperaceae* analyzed under the point of view of the type of plastid inheritance was a species of *Peperomia*, which resulted to have only maternal plastidial inheritance (Corriveau and Coleman 1988). The presence of the "short" *G3pdh* nuclear sequences may be related to a still more ancient hybridization event involving the ancestor of the Malagasy species and some ancestor of Asian origin. Also in this case probably, with Asian pollen entering in contact with the ancestor's stigma of the Malagasyan species. As a matter of fact the closest relatives to the African species *sensu lato* (including the Malagasy and the Reunion species) are Asian, with the closest species (among those here sampled) apparently from Malaysia (Figure 3 and Figure 4). Apparently interspecific hybrids can be obtained in genus *Piper* also experimentally (Vanaja et al. 2008), while the hybrid origin of several Andean species was already proposed by Quijano-Abril et al. (2006).

The presence of paralogs of *G3pdh* in angiosperms may represent a problem in several phylogenetic analysis (Hurteau and Spivack 2002); Liu et al. 2009; Sun et al 2012). However, here most of the indels were found in the introns of the gene and hence we are not able to assess the functionality of the short sequences.

The preliminary results about the chromosome numbers scored about 2n=46+-2 in *P. malgassicum* and 2n=36+-2 in *P. tsarasotrae*. The uncertainty in the counts was due to the small dimensions of the chromosomes that were observed in most of the species of the genus, together with stickiness (Samuel 1987; Samuel and Morawetz 1989), the low amount of metaphases in the root tips of the plants cultivated *in vitro* and the apparently small size of the mitotic fuse, leading to partial overlapping of many of the small chromosomes. The mitotic spindle can reach dimensions up to 60 µm (Wühr et al. 2008; Petry 2016), while in *P. malgassicum* and *P. tsarasotrae* it was about 15-20 µm (see Figure 4).

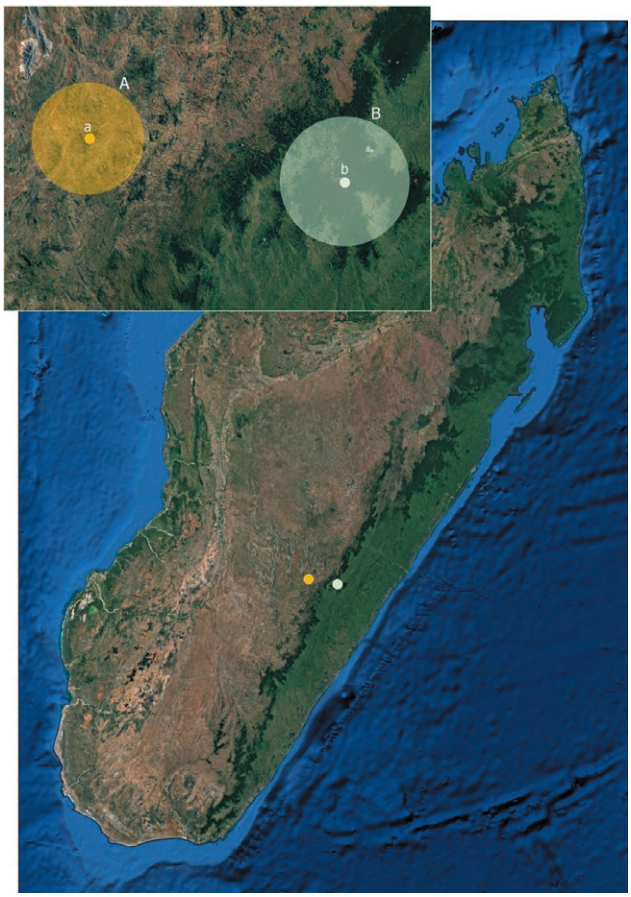

Figure 5. Geographical localization of the sampling area for *P. tsarasotrae* (yellow) and *P. malgassicum* (white). Area of the sampling campaign of 2018 (a and b) and 2019 (A and B) for *P. tsaratsotrae* (a/A) and *P. malgassicum* (b/B) respectively.

The chromosome numbers in genus *Piper* are very variable, ranging from 2n=26 to 2n=104, with some species apparently able to possess several possible chromosome numbers (Samuel 1987). Most new world species show a karyotype of 2n=26 and x=13 (Samuel and Morawetz 1989), with some exceptions having 2n=28 chromosomes (Maugini 1953). In Asia tetraploids 2n=52 would prevail (Samuel 1987). No data was available for African and Malagasy species up to the here presented results. However, the clear difference in karyotype between *P. tsarasotrae* and *P. malgassicum*, two species otherwise strictly phylogenetically related, may confirm a possible hybridization/introgression event with a species with a different chromosome number with respect to the ancestor of the Malagasyan species. As a matter of fact, also Nair et al. (1993) explained the observation of a triploid plant of *P. nigrum* (2n=78) as the result of a natural crossing between 2n=52 and 2n=104 plants.

The progeny showed a range of variation from 2n=52 to 2n=104 and production of aneuploid viable pollen (Nair et al. 1993). Hybridization may influence diversity, including gene flow from one taxon to another (introgression) and the formation of new, stable hybrid taxa and, possibly, speciation (Mallet 2007; Vallejo-Marín and Hiscock 2016).

As preliminary guess, the two different chromosome numbers of the Malagasyan species may have arisen as a consequence of hybridization of a 2n=52 species with a 2n=26 (*P. tsarasotrae*) and with another 2n=52 species (*P. malgassicum*), respectively, with following aneuploid reductions. The two species have close areals (they are almost sympatric), even if they tend to occupy different habitats, more arid *P. tsarasotrae* (not lianous habit) and more humid (lianous habit) *P. malgassicum*. Such proximity of two closely related species may be considered another possible indication of a relatively fast speciation event.

A discordance between plastid and nuclear inheritance inferred through DNA sequencing has been often related to reticulate evolution and species of hybrid origin (García et al. 2014; Stefanović et al. 2007; Aubriot et al. 2018), even if the karyological data may be a decisive evidence, it has been rarely used in relationship to the DNA sequence evidence as, for instance in Selvi et al. (2002). Here the hypothesis of an hybrid origin for the two investigated species may explain the presence of a double *G3pdh* sequence in both of them.

The relationship between the Malagasyan species and *P. guineense* with the asian species as members of *Piper* s. s. was already proposed by Jaramillo and Callejas (2004) and Jaramillo *et al*. (2008) as a result of a dispersal event, and our results do not disagree with this position. Apparently, in Africa and Madagascar the conditions leading to the wide diversification observed in South-american *Piper* (Martines et al. 2015) are lacking or less capable of influencing the speciation process.

5. CONCLUSIONS

The surprising discrepancy between the nuclear and the plastid phylogeny could be explained with an ancestral introgression event due probably to pollen contribution from an ancestor of the African mainland *P. guineense* towards the ancestor of *P. tsarasotrae*. The presence of possible paralogs of the nuclear gene *G3pdh*, clustering together with more distantly related Asian species lead to the hypothesis that a second more ancient hybridation/introgression event would have occurred between south Asian species and the ancestor of the Malagasyan species. The chromosome numbers observed in the Malagasyan species would confirm different evolutionary history.

Further studies about the karyotypes of the Malagasy species, the african *P. guineense* and *P. borbonense* will be necessary together with the investigation of the possible presence of short paralog sequences of *G3pdh* in *P. borbonense*.

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Antiproliferative analysis of aqueous extracts of cabreúva (*Myrocarpus frondosus*) on the *Allium cepa* cell cycle

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Abstract. *Myrocarpus frondosus* is widely used in Brazilian folk medicine for bronchitis, gastritis, ulcers and wound asepsis. However, there is a scarcity of scientific data proving the safe use of tea of this species or if there is any toxic effect on the human organism. This study aimed to evaluate the antiproliferative effect of aqueous extracts of cabreúva on the *Allium cepa* cell cycle. The aqueous extracts were prepared from leaves, bark and roots (dried material) of the species in two concentrations: 2.5 and 5.0 grams in 250 mL of distilled water. The aqueous extract of the leaves was obtained by infusion and the aqueous extract of the bark and roots by decoction. Distilled water was used as negative control and glyphosate 2% as positive control. Eight groups of four onion bulbs were evaluated and each group corresponded to one treatment and, in each group, 4000 cells were analyzed and the mitotic index was calculated. The results demonstrated reduction of mitotic indices in all treatments when compared to the negative control in water. The aqueous extracts of cabreúva in the studied concentrations have antiproliferative action on the *Allium cepa* cell cycle, ensuring the safe use of tea of this medicinal species.

Keywords: *Myrocarpus frondosus*, medicinal plant, mitotic index.

1. INTRODUCTION

Plants with medicinal potential have been widely used to treat various diseases and are often the only medicine available to the population (Fachinetto and Tedesco, 2009). However, indiscriminate use coupled with lack of knowledge about the medicinal species can cause harm to health (Frescura et al. 2012). The genus *Myrocarpus* is exclusively South American, being *Myrocarpus frondosus* Allemão the only species recorded in southwestern Paraguay, northern Argentina and southern, southeastern and northeastern Brazil (Lorenzi and Matos, 2008).

The species *M. frondosus* belongs to the family Leguminosae, is characterized by being a large tree plant and popularly known as cabreúva (Cabrera et al. 2012). Cabreúva is considered a relevant species because, besides being used as a medicinal plant, it is also used in reforestation of degraded areas

(Sccoti et al. 2011; Santi et al. 2017). It is popularly used for diarrhea, gastritis, ulcers, wound asepsis, has expectorant effect, anti-inflammatory and antimicrobial activity (Pereira Junior et al. 2014; Santi et al. 2017). Despite its widespread use in folk medicine, there are no scientific studies to prove its effectiveness and / or rule out possible unwanted or adverse effects from its consumption. Thus, this fact highlights the need for studies that evaluate the action of aqueous extracts of the species *Myrocarpus frondosus* on organisms.

The effects of aqueous extracts can be assessed by testing with bioindicators, which generally include subsystems of a complete organism used to identify a specific target (Leme and Marin-Morales, 2009). From the results obtained, the mitotic index is calculated. Mitotic index values are used as indicative of proper cell proliferation and measured by the *Allium cepa* plant test system. This test has been widely used as a genotoxicity bioindicator (Tedesco and Laghinghouse, 2012). The efficiency of the *Allium cepa* system is due to its proliferation kinetic characteristic, rapid root growth, large number of dividing cells, high tolerance to different cultivation conditions, permanent availability, easy handling, reduced chromosome number (2n = 16) and easy viewing under the microscope (Caritá and Marin-Morales, 2008). And from this test it is possible to monitor the effects of medicinal plant extracts, ensuring their safe use by the population in the treatment of diseases and sporadic consumption (Ubessi et al. 2019).

Considering the medicinal importance of the species *Myrocarpus frondosus* and the lack of information regarding its antiproliferative activity, the effect of aqueous extracts from leaves, bark and roots on the *Allium cepa* cell cycle was evaluated.

2. MATERIAL AND METHODS

2.1. Obtaining plant material

The plant material of the species *Myrocarpus frondosus* was collected from a population located in the west of Santa Maria, Rio Grande do Sul, Brazil, under the coordinates 29°41'23.6"S 53°50'45.3"W. The experiment was carried out at the Plant Cytogenetics and Genotoxicity Laboratory at the Federal University of Santa Maria (UFSM).

2.2. Preparation of aqueous extracts

For the preparation of aqueous extracts leaves, bark and roots (dried material) were used in two concentrations: 2.5 and 5.0 grams (g) in 250 mL of distilled water. The leaves were placed in a container containing boiling water, remaining infused for 10 minutes. The extracts of the bark and roots of cabreúva were prepared by decoction in a period of 10 minutes. All extracts after 10 minutes were strained and stored until room temperature.

2.3. Allium cepa test

The *Allium cepa* test was developed at the Plant Cytogenetics and Genotoxicity Laboratory (UFSM) and organized into eight groups of four onion bulbs, which were placed for rooting in distilled water for a period of 72 hours. Distilled water was used as negative control and glyphosate 2% as positive control. The evaluated treatments are described in Table 1.

After rooting, the bulbs remained in contact with the treatments described in Table 1 for a period of 24 hours. Time lapse mentioned, the roots were detached from the bulbs and fixed in Carnoy 3:1 (ethanol: acetic acid) for 24 hours at room temperature. Soon after, the roots were placed in 70% ethanol and stored under refrigeration until blades preparation.

2.4. Preparation of blades

Two blades per bulb were made, and 500 cells per blades were analyzed, totaling 4000 cells per treatment. The preparation of the blades was performed according to the crushing technique (Guerra and Souza, 2002). In this procedure the roots were washed in distilled water and hydrolyzed for 5 minutes in HCL 1N at room temperature. They were then washed again in distilled water with removal of the meristematic region and stained with acetic orcein 2%. The analysis of these blades was performed under a 40X magnification optical microscope, taking into account the phase of the cell cycle in which the cells were present, such as interphase, pro-

Table 1. Treatments evaluated in the *Allium cepa* test.

Treatments
T1. Distilled water - Negative control.
T2. Glyphosate 2% - Positive control.
T3. Infusion of 2.5 g of dried leaves.
T4. Infusion of 5.0 g of dried leaves.
T5. Decoction of 2.5 g of dried bark.
T6. Decoction of 5.0 g of dried bark.
T7. Decoction of 2.5 g of dried roots.
T8. Decoction of 5.0 g of dried roots.

phase, metaphase, anaphase and telophase. To calculate the mitotic index was considered the sum of the number of cells in prophase, metaphase, anaphase and telophase, divided by the total number of cells observed (Sehgal et al. 2006; Vieira et al. 2009). The result is presented as a percentage.

2.5. Statistical analysis

The data related to the mitotic index were submitted to the Chi-square test ($\chi 2$) with the aid of the statistical program BioEstat 5.3 (Ayres et al. 2007).

3. RESULTS

The results found for the controls and treatments evaluated in relation to the mitotic index (MI) are presented in Table 2. The cells observed for the counting and evaluation of the treatments were in interphase and cell division (Figure 1). The negative control (T1) presented higher MI (6.12%), differing significantly from all other treatments evaluated. The positive control (T2) also differed statistically from the negative control, showing lower MI, thus confirming its antiproliferative action. Results differed significantly between treatments and controls (Table 2). In the comparison between the negative control (T1) (MI= 6.12%) and the positive control (T2) (MI= 4.55%), there was a significant difference and decreased IM, which indicates inhibition of cell division.

Positive control (T2) differed significantly from treatments with bark extract (T5 and T6) and root extract (T7 and T8), and the extracts further inhibited cell division in relation to glyphosate. Comparing the treatments of aqueous extracts of leaves at both concentrations (T3 and T4) with glyphosate treatment (T2), there was the same behavior, as the MI did not differ statistically, showing a decrease in cell division. Treatments with aqueous extracts of dried bark were significantly different. The extract with higher concentration (T6) strongly inhibited cell division, being the lowest mitotic index observed (2.8%). In relation to treatments with dried root extracts, the two differed statistically from each other, but unlike what occurred with treatments with bark extracts, it was the treatment with the lowest concentration (T7) that most reduced the MI (2.95 %).

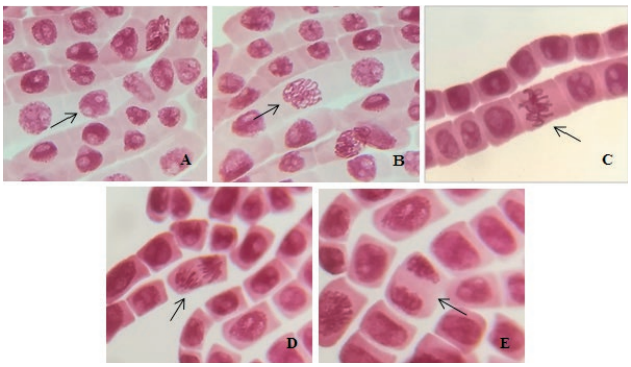

Figure 1. Cell cycle phases of *Allium cepa* with interphase cell and division cells. Interphase (A). Prophase (B). Metaphase (C). Anaphase (D). Telophase (E). Scale 10 µm.

Table 2. Interphase, cell division and mitotic index in *Allium cepa* cells.

Treatments	Cells analyzed	Interphase cells	Dividing cells	MI (%)
T1. Distilled water	4000	3.755	245	6.12 a*
T2. Glyphosate 2%	4000	3.818	182	4.55 b
T3. 2.5 g de DL	4000	3.811	189	4.72 b
T4. 5.0 g de DL	4000	3.816	184	4.60 b
T5. 2.5 g de DB	4000	3.853	147	3.67 c
T6. 5.0 g de DB	4000	3.888	112	2.80 d
T7. 2.5 g de DR	4000	3.882	118	2.95 d
T8. 5.0 g de DR	4000	3.858	142	3.55 c
Total	32000	30681	1319	-

*Averages followed by the same letter in the column do not differ from each other by the $\chi 2$ test at a 5% error probability level. DL= dried leaf; DB= dried bark; DR= dried root; MI= mitotic index.

4. DISCUSSION

All treatments differed significantly from the negative water control (T1) demonstrated by the decrease in MI values. This means that there has been a reduction in cell division of the meristematic cells of *Allium cepa*. This decrease indicates antiproliferative activity of aqueous extracts from the leaves, bark and roots of cabreúva at both concentrations used. The observed cells of the onion bulb root that were submitted to the lowest concentrations of extracts prepared by infusion (T3) and decoction (T5) obtained a cellular stimulus, resulting in the increase of the IM compared to those with higher concentrations (T4 and T6). This means that the higher concentrations of leaf and bark extracts caused the reduction of MI. In contrast, the aqueous extract from dried roots, at higher concentration (T8), increased the MI compared to the lower concentration treatment (T7). Therefore, aqueous extracts of dried leaves and bark

show antiproliferative capacity, especially at high concentrations.

By studying *Luehea divaricata* in two populations and at concentrations of 6 g L^{-1} and 30 g L^{-1}, Frescura et al. (2012) also observed this same behavior regarding cell proliferation. The higher concentration led to a decrease in MI values, concluding that there was an increase in antiproliferative capacity with increasing concentration. Coelho's research (2013), analyzing two populations of *Echinodorus grandiflorus* at concentrations of 6 g L^{-1} and 24 g L^{-1}, also found increased antiproliferative activity at the highest concentration, except for a commercial extract treatment. In this study, regarding the aqueous extracts of leaves, there was no significant difference between the concentrations studied (Table 2). Similarly, Kuhn (2015), when analyzing the *Peltodon longipes* species, observed that leaf extracts at different concentrations (5 g L^{-1} and 15 g L^{-1}) did not differ significantly. Other tree species such as aroeira (*Myracrodruon urundeuva*) (Trentin et al. 2013), graviola (*Annona muricata*), ipê-roxo (*Handroanthus impetiginosus*) (Melo et al. 2010), angico-branco (*Anadenanthera colubrina*) (Lima et al. 2014) and pau-ferro (*Libidibia ferrea*) (Guerra et al. 2017) also have antiproliferative activity found in their extracts from various plant parts, such as bark and leaves. Some species even belong to the same family as *Myrocarpus*, reaffirming the results obtained in this study.

Myrocarpus frondosus bark extracts had an MI of 3.67% at a concentration of 2.5 g, while the highest concentration had an MI of 2.8%, reducing cell proliferation when the concentration of the extract increased. There was a significant difference between the two concentrations (Table 2). However, other authors, such as Frescura et al. (2012), studying the species *Luehea divaricata*, did not observe significant differences between bark extracts at concentrations 32 g L^{-1} and 160 g L^{-1}. Considering the results observed for the root decoctions at different concentrations, there is a difference between the concentrations, because the inhibition of cell division was smaller as the extract concentration increased (Table 2). Studying aqueous extracts of *Lavandula angustifolia* roots at a concentration of 0.29 g in 250 mL of distilled water, Freitas et al. (2016) found antiproliferative potential compared to controls. However, Rodrigues et al. (2017), analyzing the same species in higher concentration (3.75 grams in 200 mL of distilled water) observed proliferative potential. The increased concentrations of aqueous extracts of *Lavandula angustifolia* roots induced an increase in the proliferative capacity of the species, a behavior also observed in this study with *Myrocarpus frondosus* roots.

When studying the roots of *Myrocarpus frondosus in vivo* and *in vitro*, Bottamedi et al. (2018) found antioxidant and anti-inflammatory activity which were attributed to the presence of flavonoids and phenolics. In addition, the analyzes performed on the essential oil of *Myrocarpus frondosus* leaves showed the presence of α-thujene, α-pinene, sabinene, β-pinene, mircene, p-cymene, limonene, β-bourbonene, β-caryophyllene, D- germacrene, bicyclogermacrene, spatulenol and globulolem, with predominantly β-pinene and bicyclogermacrene (Cabrera et al. 2012; Santi et al. 2017). In other studies with *Echinodorus grandiflorus* (Coelho, 2013), *Baccharis trimera*, *Baccharis articulata* (Fachinetto and Tedesco, 2009), *Caesalpinia echinata* (Bastos et al. 2011) and *Myracrodruon urundeuva* (Romano et al. 2013) were also found substances like flavonoids and tannins. These chemical components were attributed to the antiproliferative capacity presented by the researched plants mentioned above. Flavonoids exert a broad spectrum of health-beneficial biological activities, including the antiproliferative effect on cancer cells (Gibellini et al. 2011; Tsai et al. 2016), reaffirming the results obtained with the species *Myrocarpus frondosus*.

5. CONCLUSION

The aqueous extracts of leaves, bark and roots of *Myrocapus frondosus* at concentrations of 2.5 and 5.0 grams have antiproliferative effect on the cell division of *Allium cepa* meristematic cells.

Aqueous extracts from the 5.0 gram *Myrocapus frondosus* bark exhibit high antiproliferative capacity.

The antiproliferative activity found in the species *Myrocapus frondosus* may be associated with the presence of flavonoid and phenolic compounds in the plant tissue composition of the species.

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Exploration of diversity and distribution of cytotypes of *Saccharum spontaneum*, a wild species of sugarcane, in India

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Abstract. The present investigation was undertaken to examine the geographic distributions of cytotypes of *Saccharum spontaneum* L., wild species of sugarcane, in India. New chromosome determinations has been done for 524 accessions collected mainly from four ecological zones, West, East, North and North-east, of the country. A detailed evaluation of cytogeographic pattern of *S. spontaneum* has been done with these clones along with the clones in which chromosome data is already available. Twenty six cytotypes ranging from 2n=40 (8x) to 2n=112 (14X) has been identified in *S. spontaneum* from India. Gangetic valley of Sub Himalayan region and deltaic region of South-east zone can be considered as regions of cytogeographic interest with largest concentration of different chromosome numbers. North-east region of the country appears to have had a higher evolutionary activity in this species which is evidenced by the occurrence of multiple cytotypes and sympatric growth with other species and genera. The overall cytogeographic pattern of *S. spontaneum* includes the incidence of mixed polyploidy, aneuploidy, sympatric with different ploidy levels and disjunct distribution of some cytotypes indicate that this species likely to have had multiple independent origin in different parts of India.

Keywords: *Saccharum spontaneum*, Chromosome number, Mitosis, India, Cytogeography, Polyploidy, Cytotype diversity.

INTRODUCTION

The genus *Saccharum* comprises of six species Viz., *Saccharum officinarum* L., *Saccharum barberi* Jesweit, *Saccharum sinense* Roxb. Amend. Jeswiet and *Saccharum edule* that are cultivated and *Saccharum robustum* Brandes and Jesweit ex Grassl and *Saccharum spontaneum* L. that are wild. Among these species *S. spontaneum* was subjected to detailed studies in India due to its wide distribution and high variability in morphology and chromosome number and most importantly its contribution towards the genetic improvement of cultivated sugarcane by conferring resistance to major diseases, providing vigor and hardiness for increased abiotic stress tolerance (cold and drought), increased tillering and improved ratoonability.

The commercial success of the early interspecific hybrids involving *S. spontaneum* generated interest in the collection and utilization of wild sugarcane germplasm. India is one of the major centers of diversity for *S. spontaneum*. It has a wider distribution throughout the country, from sub – Himalayan region to Peninsular India. The sugarcane germplasm collection from India dates back to 1912. During that time Dr. C.A. Barber collected *S. spontaneum* clone Coimbatore which later become the male parent of the first sugarcane variety, Co 205. Further collections of *S. spontaneum* were made by Sir Venkataraman and Dr. Janakiammal. Later the 'Spontaneum Expedition Scheme (SES)' sponsored by the Indian Central Sugarcane Committee was operated during 1948-1957, with the objective of collecting the wild *Saccharum* germplasm from the distributional areas in the country and outside. Collection efforts were revived during 1980s and six more explorations were conducted to North Eastern states of India during 1981-1990. Further exploration for collection of *Saccharum* germplasm was conducted under the National Agricultural Technology Project on Plant Biodiversity (NATP-PB) during 1999-2004. Under this programme explorations were conducted in Arunachal Pradesh, Mizoram, Orissa, Andra Pradesh, Karnataka, Tamil Nadu, Kerala and Andaman and Nicobar Islands (Nair, 2013). Explorations are being continued thereafter under the Institute programmes and the states of India like Tripura (2005), Meghalaya (2006), Gujarat (2007), Rajasthan (2008), Himachal Pradesh (2009), Uttarakhand (2009), West Bengal (2010), Nagaland and Manipur (2011), Maharashtra (2015), Punjab and Haryana (2016), Jharkhand (2017), West Bengal and Sikkim (2018 and Assam (2019) were explored during the subsequent years.). The explorations covered all states of the country and the cytogenetic studies revealed that the *S. spontaneum* germplasm collected represent the whole range of cytotype diversity present in the respective state.

Extensive cytological studies have been conducted in *S. spontaneum* accessions available in germplasm collection at ICAR-Sugarcane Breeding Institute, Coimbatore, India. Natural occurrence of around 31 cytotypes in *S. spontaneum* ranging from 2n=40-128 was established from these studies of accessions from in and out of the country (Janakiammal 1939; Panje and Babu 1960; Mehra and Sood 1974; Sreenivasan 1975; Kandaswamy et al. 1983; Sreenivan and Sreenicasan 1984, 1994; Praneetha and Nair, 2005; Sobhakumari and Mallika 2007; Sobhakumari 2009; Sobhakumari 2013 and Sobhakumari and Stanly 2017

Survey on the geographical distribution of different cytotypes of *S. spontaneum*, which were identified till 1960, has been conducted by Panje and Babu (1960) and they could substantiate the proposal of Parthasarathy and Rao (1946) that the Indian sub-continent (including Nepal, Bangladesh, Pakistan and Sri Lanka) has mostly low numbers ranging 2n=40-80. After this survey the geographic distribution of the cytotypes of *S. spontaneum* has not been analyzed critically. A detailed knowledge of the geographic distribution of ploidy variation within the species comprising of polyploid complexes is critical to our understanding of the history and evolution of such complexes. In this study an elaborative cytological analysis of accessions of *S. spontaneum* from different states of India is used to resolve its cytogeographic pattern in the country. In particular, the study will address the following questions. (1) What is the ploidy variation of *S. spontaneum* across its distribution range (based on the representative samples collected from different states of India), (2) Is this variation geographically/ecologically structured (3) Where is the center of ploidy level diversity (4) How many cytotypes are available for this species in India and its role in the evolution of euploids and aneuploids in the same species.

MATERIALS AND METHODS

The materials included in the study are collections about 524 in number, from different states of the country which were made during 2001-2017. The details of the number of clones used in the study from different states of the country are given in Table 1. Most of these clones were documented in S. spontaneum catalogues (Sreenivasan et al. 2001, Nair et al. 2013). The clones were numbered based on the following method. IND represents the country of origin (INDIA), next number (01-17) represents the year of collection and the last number represents the accession number. From the place of origin the materials were collected as clumps/ suckers and were established at germplasm fields of ICAR-Sugarcane Breeding Institute, Coimbatore after proper quarantine. For cytological studies small parts of clumps were planted in pots to collect root tips. For mitotic studies the root tips were pretreated with saturated solution of alpha bromo naphthalene at 4°C for 2h. Then the washed materials were fixed in 3:1 (alcohol: acetic acid) fixative overnight. Washed root tips were hydrolyzed in 1N HCl and stained in 1% acetocarmine. A minimum of 10 well spread metaphase plates were used for chromosome count and photographed in Carton 402T microsystem.

The cytologically analyzed 524 accessions of *S. spontaneum* include 478 new determinations of chromosome number from the clones collected under Institute project

Table 1. Details of *S. spontaneum* clones cytologically analyzed.

Clone	Year of collection	State	No. of clones studied
IND 01	2001	Orissa	3
IND 02	2002	Andhra Pradesh	6
IND 03	2003	Andaman	24
IND 04	2004	Mizoram	39
IND 05	2005	Tripura	13
IND 06	2006	Meghalaya	16
IND 07	2007	Gujarat	25
IND 08	2008	Rajasthan	11
IND 09	2009	Himachal Pradesh & Uttaranchal	45
IND 10	2010	West Bengal	26
IND 11	2011	Nagaland & Manipur	91
IND 15	2015	Maharashtra	39
IND 16	2016	Punjab & Haryana	88
IND 17	2017	Jharkhand	52
IND 90	1990	Arunachal Pradesh	19
SES,IND81 & R collections	1954, 1981, 1937	Madhya Pradesh, Bihar and AP	27

(2001-2017), 19 clones collected from Arunachal Pradesh during 1990 (IND 90 clones) and 27 clones which were collected under SES programme and R collections done by Dr Janakiammal. The clones recently analyzed (478) were mostly covered the West zone, East zone, North zone and North-East zone of India. In order to cover the whole country the earlier cytological reports of *S. spontaneum* were also considered for cytogeographic survey. For this purpose the clones studied by Panje and babu (1960), Sreenivasan (1975), Sreevivasan and Sreevivasan (1984 and 1994), Praneetha and Nair (2005), Sobhakumari and Mallika (2007), Sobhakumari (2009), Sobhakumari (2013) and Sobhakumari and Stanly (2017) were included. All the chromosome number data have been pooled together and a superimposed map of on the cytogenetic distribution of *S. spontaneum* in India has been resolved. To avoid confusion the chromosome numbers of all the clones are referred by their 2n numbers, although most of the earlier determinations have been made on pollen mother cells (meiosis). To avoid hinder during discussion the authorship references are not given each time when a cytotype is mentioned.

RESULTS AND DISCUSSION

Among the *Saccharum* species, the wild *S. spontaneum* was subjected to detailed studies in India due to its wide distribution, extensive variability in morphology and chromosome number, and most importantly its use in genetic improvement of cultivated sugarcane. Its somatic chromosome number as on now known extends from 2n=40-128 with basic chromosome number x=8. In the present study we examined the geographic distributions of cytotypes of *S. spontaneum* in different parts of India. For the present study India has divided into 6 geographic zones viz., (1) South zone, (2) West zone, (3) East zone, (4) North zone, (5) North-East Zone and (6) Central zone. The cytotype of 524 accessions were determined based on somatic chromosome count which were mainly distributed in four zones viz. (1) West zone, (2) East zone, (3) North zone and (4) North-East zone. Few clones were studied from Peninsular India and also from Andaman islands.

West zone

This zone includes Rajasthan, Gujarat and Maharashtra. Three cytotypes, 2n=64, 72 and 80 were common in these states. Of 34 *S. spontaneum* clones collected from Gujarat, 25 were studied cytologically. Majority of the clones were 2n=80 (80%) and many of them were morphologically dwarf. Surprisingly only one clone, IND 07-1486, showed 2n=64 chromosomes. With two clones of 2n=72 two aneuploids of 2n=74 and 76, one each, were also present in Gujarat. Though Rajasthan is a nearby state, its collection was having only one clone, IND 08-1502, with 2n=80. Other than this 2n=64 and 2n=72 cytotypes were also present in low frequencies. This state reports only cytotypes with multiples of eight, i.e., 8x, 9x and 10x. In Maharashtra out of 41 clones collected, 39 clones were cytologically analyzed. Six cytotypes, 2n=60, 62, 64, 66, 72 and 80 were identified from this collection. Majority of the clones were with 2n=64 (77%). Other two euploids present in the collection were 2n=72 (9x) and 2n=80 (10x). Few aneuploids like 2n=60, 62 and 66 were also present with polyploids that of multiples of eight.

As the range of somatic chromosome number reported in *S. spontaneum* is 2n= 40-128, from west zone of India intermediate numbers of this range were reported. Majority of the clones were 2n=62, 72 and 80. As far as the distribution of the cytotypes concerned a specific segregation could observe in the states of this zone. In Gujarat majority of the clones were with 2n=80 (80%). This was an unusual occurrence while compare to other

states of the country where mixed ploidy was observed. This finding contrast with the report of Panje and Babu (1960) where they have identified 2n=80 chromosome forms restrictedly in Nepal, Assam and the Western Ghats of India. The nearest state Rajasthan was having only one clone with 2n=80 cytotype and also absent in aneuploids. This indicates that 2n=64, 72 and 80 cytotypes are cytologically stable with normal meiosis and normal chromosome segregation.

East zone

This zone covers the states Bihar, Jharkhand, West Bengal and Odisha. The Clones that studied from Bihar were collected during 1950 (SES) and 1981 (IND 81). Most of them were 2n=64 cytotypes and one clone each of 2n=56 and 2n= 90 cytotypes were also there. Jharkhand collections were done recently in 2017. From this collection 52 clones were subjected to cytological analysis. Cytotype range identified in this collection was 2n=40-72. Among this, lower cytotypes i.e., 2n=54 and 56 were in higher frequency. The reported lowest chromosome type of *S. spontaneum* has identified in Jharkhand collection, i.e., 2n=40 (IND 17-1852). A total of 8 cytotypes were identified from this state in which aneuploids were lesser in number while compared to euploids. In west Bengal collection (IND 10) only four cytotypes were identified, i.e., 2n=60, 64, 70, 72. Out of 26 clones analyzed cytologically it was found that majority of them were with 2n=60 (73%). The euploids (multiples of 8) like 2n=64 and 72 were present only in low frequency. From Odisha three cytotypes were identified as 2n=52, 64, 112. IND 01-1157 (2n=112) from Odisha is the *S. spontaneum* clone with highest number reported from the present study.

From the four states of East zone of India, the Lowest chromosome number, 2n=40, and the highest chromosome number, 2n=112 were reported. These extreme types were only in low frequency, i.e., one in each. West Bengal was showing a peculiar cytotype, i.e., 2n= 60 in high frequency. This cytotype was identified from other states also, but in low frequency. In Bihar the lowest number was 2n=56 and highest number was 2n=90. As very few clones were studied from this state at present it is not sensible to conclude the position of *S. spontaneum* cytotypes existing in this state. There are chances for having the intermediate chromosome numbers from natural hybridization. To substantiate this view earlier reports revealed that from Bihar 14 cytotypes were identified by analyzing more number of accessions of *S. spontaneum* (Sreenivasan and Sreenivasan, 1994). Jharkhand was having low cytotypes like 2n= 40, 56, 64, 72 (euploids) and this may be the cause of the existence of other aneuploids in this region due to intraspecific natural hybridization. Panje and Babu (1960) reported the possibility of existence of cytotypes with different range of chromosome numbers in the region where low cytotypes are abundant. Odisha was having the highest chromosome number 2n=112 and lowest chromosome number 2n=52. In this study only few clones from Odisha has been included and the picture of chromosome survey in this state is not adequate to come to the conclusion about its cytogeographic pattern. This will be clearer in the later part of the study where all previous reports on cytological analysis of *S. spontaneum* from the same area were considered.

North zone

In this zone *S. spontaneum* has been collected from four states namely Himachal Pradesh, Uttaranchal, Punjab and Haryana. During 2009 a combined collection has been done from Himachal Pradesh and Uttaranchal. All the collected clones of *S. spontaneum*, 45 cloones, were subjected to cytological analysis. Majority (71%) of them comes under the cytotype category 2n=54 and 2n=56. More number of clones (6 clones) were identified from here with 2n=40. Other cytotypes present in Himachal Pradesh and Uttaranchal were 2n=60, 64 and 72. Surprisingly only one clone was with 2n=64 though it is considered as the most prevailing cytotype in India. During 2016 a combined collection has been made from Punjab and Haryana. High chromosomal diversity has been revealed by studying 88 accessions collected from these states. In this region twelve cytotypes were identified such as 2n=40, 48, 50, 52, 54, 56, 60, 64, 70, 72, 74 and 76. As in Himachal Pradesh and Uttaranchal the lower cytotypes 2n=54 and 56 were more in Punjab and Haryana also. It covered around 60% of the whole collection. All the other cytotypes were present only in less than 8%.

Very clear demarcation in the chromosome number of *S. spontaneum* has been shown by North zone of India from other zones because of the high frequency of low chromosome number types like 2n=40, 54 and 56. 2n=64 cytotype was in less frequency in this zone. In Punjab and Haryana among 12 cytotypes 2n=40, 48, 56, 64, and 72 were euploids (multiples of basic chromosome number 8) with chromosome constitution 5x, 6x, 7x, 8x and 9x respectively. Others were aneuploids and may be originated from intraspecific hybridization among the different ploidy cytotypes at the place of origin itself. Though in Punjab and Haryana six clones of 2n=64 were available, only one clone was with 2n=64 in Himachal

Pradesh and Uttaranchal collection. It has been reported that inter and intraspecific natural hybridization are responsible for the existence of extensive euploidy and aneuploidy in *S. spontaneum* (Janaki Ammal, 1936; Janaki Ammal and Singh, 1936; Raghavan, 1953; Kandasami. 1961a; Bremer, 1961a; Kandaswamy and rao, 1963; Sreenivasan and Jagthesan, 1973). Analysis on the evolutionary origin of different cytotypes of Punjab and Haryana collection revealed its independent as well as multiple origins (data not published).

North-East zone

In this zone the cytological analysis has been done for *S. spontaneum* clones collected from the states Sikkim, Meghalaya, Tripura, Mizoram, Manipur, Nagaland and Arunachal Pradesh. In Sikkim only one cytotype, i.e., 2n=64 was identified. From Meghalaya 16 clones were studied and four cytotypes, 2n=60, 64, 70 and 80 were identified. Of this 2n=64 (8x) was showing majority (59%) and next to it was 2n=80 (10x). The other two aneuploids would have been derived as a result of intraspecific hybridization of euploids. These aneuploids, 2n=60 and 2n=70, were in 12% and 16% respectively. In 2005 collections of were made from Tripura. Thirteen clones were cytologically analyzed and chromosome number has been determined by root tip mitosis. It was found that majority of the clones were with 2n=64 (31%). The other cytotypes available in Tripura were 2n=80 (23%), 2n=72 (23%). These clones were with chromosome numbers that were the multiples of 8. Two types of aneuploids identified from Tripura were 2n=60 and 52. They were less in percentage, 15% and 8% respectively. Mizoram collections were made during 2004. Thirty nine clones were cytologically analyzed from this collection and 12 cytotypes were identified. They were 2n= 56, 58, 60, 62, 64, 70, 72, 76, 78, 80, 88 and 90. While considering eight as the basic chromosome number of *S. spontaneum*, 2n=56, 64, 72, 80 and 88 were polyploids with chromosome constitution 7x, 8x, 9x, 10x and 11x respectively. 2n=64 (26%) and 2n=80 (28%) cytotypes were in majority in this state and next to this was 2n=56 (13%). All the other cytotypes were in less frequency and it was found that all together the nine cytotypes covered 33% of the total clones studied. Combined collection was made from Manipur and Nagaland during 2011 and cytological analysis has been done in 91 clones of *S. spontaneum*. Nine cytotypes, 2n=54, 56, 58, 60, 64, 70, 72, 74, and 80 were identified from these states. Majority of the collection (62%) was with 2n=64 and next to it was 2n=80 (22%). All other cytotypes were in low frequency. Nineteen clones were cytologically analyzed from Arunachal Pradesh and six cytotypes were identified as 2n=54, 56, 58, 62, 64 and 90. Majority of the collections were with 2n=64. All other cytotypes present in this collection were in less number. From Arunachal Pradesh one clone, IND 90-755, was with 2n=90 which is a rare cytotype with high chromosome number occurred in India.

From the result of cytological studies conducted in *S. spontaneum* clones from the states of North East region of India make us to recall the statement of Dr. C.A. Barber that "one of the keys which can unlock the question of ancestral sugarcane forms is concealed in North India". In the present study 179 clones of *S. spontaneum* from different states of North East Zone were cytologically analyzed. These clones have been collected from diverse habitats and different altitudes. Fourteen cytotypes including 2n=52, 54, 56, 58, 60, 62, 64, 70, 72, 74, 76, 80, 86 and 90 were identified from this study. This revealed that this region is showing high ploidy diversity for its cytotypes. This high genetic variability is due to its high compatibility between the groups and even with other related genera and species. Though we could see variable numbers with euploids of 7x, 8x, 9x, 10x and many intermediate aneuploids it was interesting to note that the lowest chromosome numbers in this species, 2n=40 and 48, were absent in this region. In earlier report the cytotype 2n=40 has been reported from Sikkim and Arunachal Pradesh (Sreenivasan and Sreenivasan, 1994). They observed that irrespective of the climatic condition prevailing in the distributional area, all clones with 2n=40 were short, saturated, less cane forming with very narrow leaves due to reduction of lamina to midrib. Contradictory to this 2n=80 cytotypes in most of its morphological characteristics it resembles *S. barberi*. In the present study determination of chromosome numbers from the recent collections have revealed new cytotypes. Earlier reports of the occurrence of low chromosome types in Sikkim and Arunachal Pradesh, occurrence of other related species and genera and existence of natural hybrids with different chromosome numbers in the North east region provides further evidence for the evolutionary significance of this zone. Due to the existence of all members of "*Saccharum* complex", overlapping of flowering time, and high compatibility between the species makes this area much evolutionary significant as far as sugarcane is concerned.

During 2003 collection of *S. spontaneum* has been done in Andaman Islands and the cytological analysis showed that only 2n=64 (8X) and 72 (9x) were available here.

The essential first step when gaining insight into

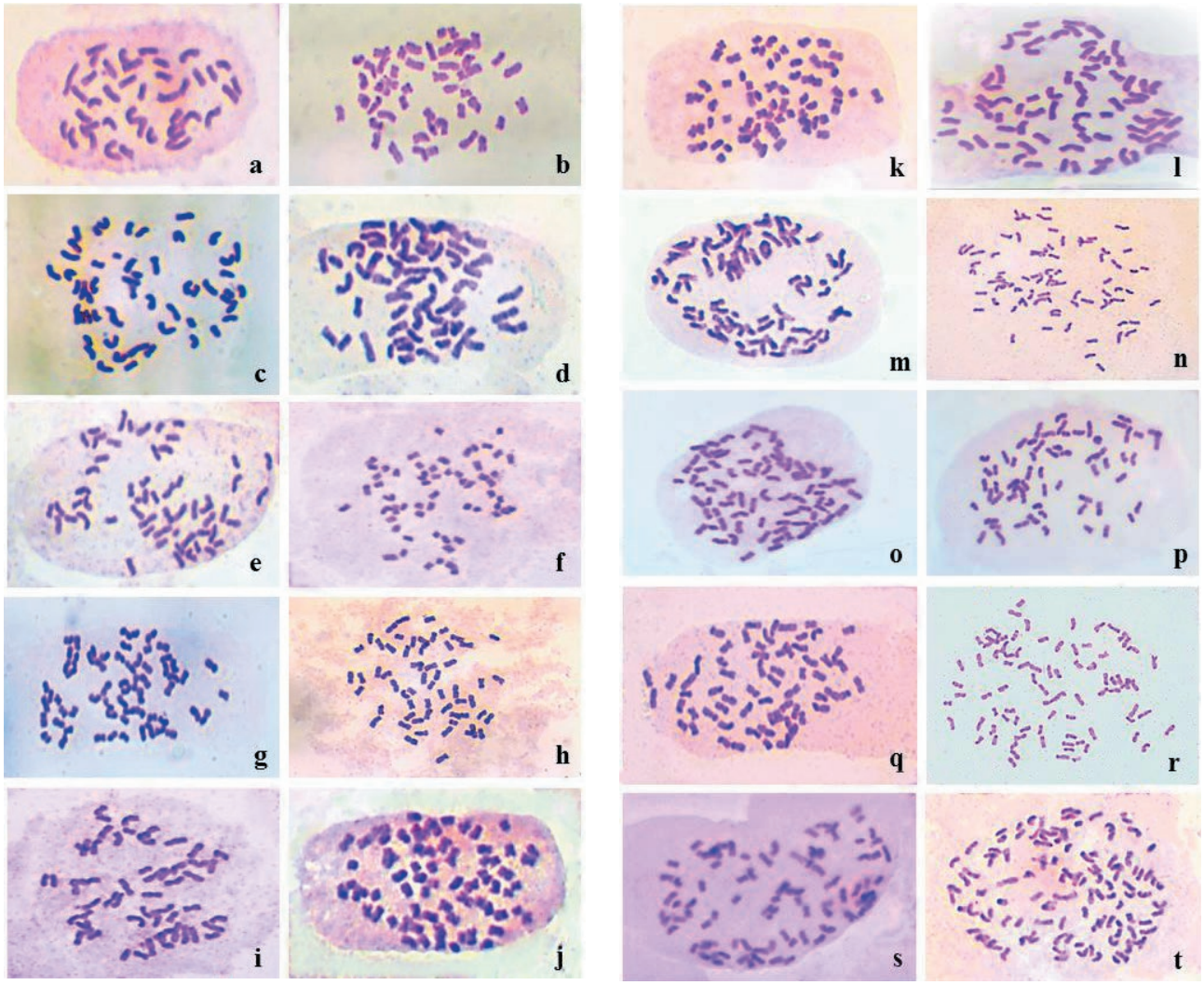

Figure 1. (a) – (t) Somatic chromosomes in different cytotypes of *S. spontaneum* a) IND 16-1812 (2n=40), b) IND 16-1792 (2n=48), c) IND 11-1606 (2n=54), d) IND 11-1604 (2n=56), e) IND 11-1614 (2n=58), f) IND 10-1574 (2n=60), g) IND 89-754 (2n=62), h) IND 11-1610 (2n=64), i) IND 01-1156 (2n=52), j) IND 17-1862 (2n=66), k) IND 17-1866 (2n=70), l) IND 15-1741 (2n=72), m) IND 10-1585 (2n=74), n) IND 09-1552 (2n=76), o) IND 07-1457 (2n=80), p) IND 08-1494 (2n=64), q) IND 03-1312 (2n=64), r) IND 04-7353 (2n=86), s) IND 90-775 (2n=90), t) IND 01-1157 (2n=112).

the evolution of polyploid is cytogeography, the study of cytotype diversity and its past and predicted future distribution patterns. Knowledge of cytotype distribution pattern usually reveals phenomena such as environmental segregation or productive isolation of cytotypes (Rejlova et al., 2019). While superimposed the map of Figure 1 which explained the state wise chromosome numbers of recently collected *S. spontaneum* clones from different zones of India with the earlier reports on the same aspect, we are getting a comprehensible picture of the geographic distribution of different cytotypes of *S. spontaneum* throughout Indian sub-continent. In Figure 2 the merged map with distribution details of so far reported cytotypes of *S. spontaneum* has been given. In Figure 3 the distribution pattern of 26 cytotypes of *S. spontaneum* in six geographical zones of India has been specified.

The earlier report says that in the case of *S. spontaneum* wherever the low cytotypes occur there is a certain concentration of other chromosome numbers also (Panje and Babu, 1960). The cytotype distribution pattern of North and North-East zones substantiate this statement by having maximum number of cytotypes that in the country reported. Although there are many incidents of polyploid coexistence in nature, the minority cytotype exclusion hypothesis predicts that mixed

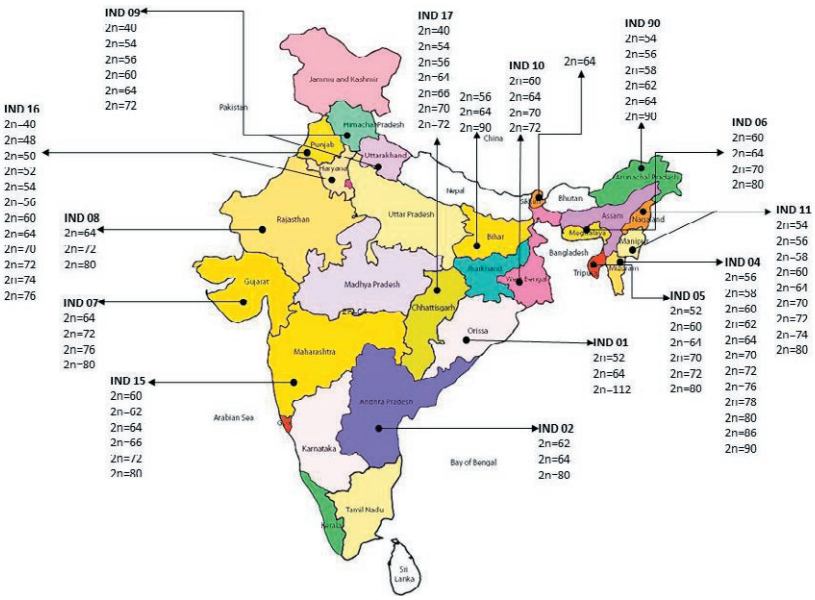

Figure 2. Distribution of *S. spontaneum* cytotypes (from 2001-2017 collection) in different states of India.

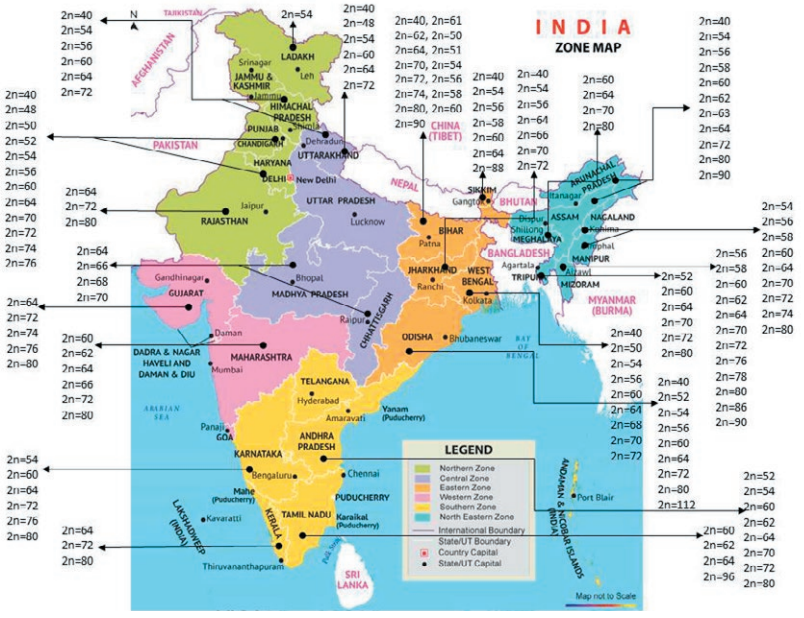

Figure 3. Superimposed map showing distribution of *S. spontaneum* cytotypes (so far reported) in different states of India.

cytotype population will eventually lose one cytotype (Levin, 1975). This may be the reason for absence of 2n=40 and 2n=48 in majority of the states of North east zone even though around 17 cytotypes were available in this zone.

A wide ranging track could be marked to incorporate all lowest chromosome number cytotypes (2n=40, 48, 54 and 56). These cytotypes could be identified from two areas which covers the entire northern sub Himalayan plain of Ganga and Yamuna river and this extended to the south east coast which encircled the deltas of Mahanadhi, the Godavari and Krishna. This identified area is coincides with the geographic pattern explained by Panje and Babu (1960). While considering the geographic pattern of different cytotypes of *S. spontaneum* in India the above mentioned track is of special interest

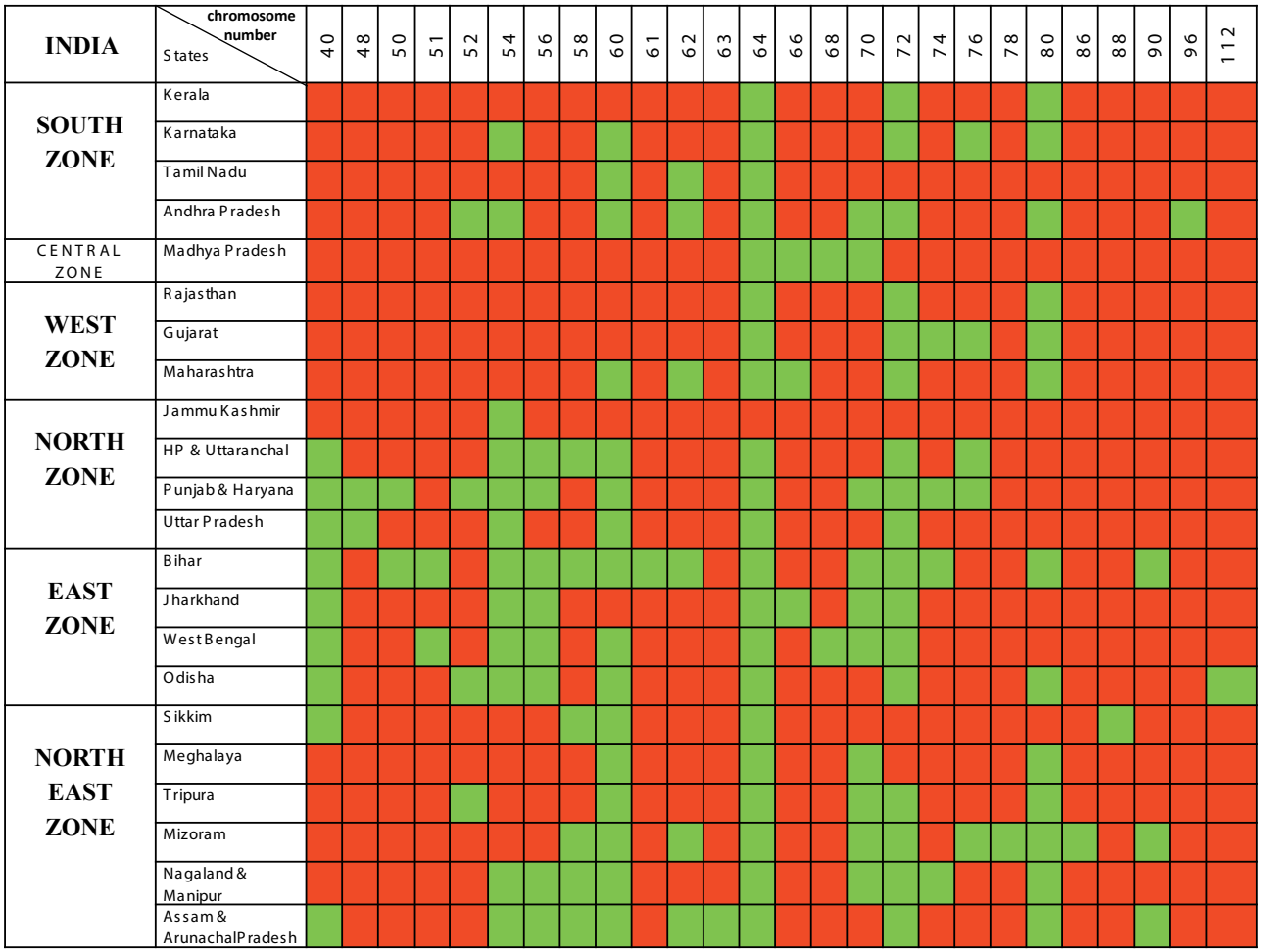

Figure 4. Distribution pattern of *S. spontaneum* cytotypes in different geographic zones of India.

because it is found to have as many as 18 out of 26 cytotypes reported from India.

The cytotype of 2n=64 has the widest distribution in India (Figure 3). This may be due to its better competitive ability compare to other cytotypes. The lower part of the peninsular India which consists of Tamil Nadu and Kerala was having more number of 2n=64 types than in North India. In Kerala only euploids like 8x, 9x and 10x were present and no intermediate chromosome numbers. Given the geographical separation and habitat similarity among cytotypes, mixed-ploidy populations may be transitional and subject to the forces of minority cytotype exclusion which lead to pure-ploidy populations (Castro et al, 2018). In most of the states with 2n=64 types, its aneuploids, 2n=60, 62, and 66, were also observed in low concentration. Mating between intraspecific cytotypes which are at the levels of ploidy often produce offsprings with odd number of genomes or imbalanced ploidy having lower fitness than those produced by individuals of same cytotype. Generally, the odd polyploids show meiotic abnormalities and consequent decreased viability of gametes leading to little success in existence (Rani et al., 2015). In contrast to this in West Bengal relatively higher concentration of (73%) 2n=60 cytotype was observed which seems to be distinct from other states.

2n=54 cytotype groups are more confined to the North zone of the country. Its relative concentration is more in Himachal Pradesh, Uttaranchal, Punjab and Haryana. This is the only chromosome number so far reported from Jammu and Kashmir (SES 352, 2n=54). Though many cytotypes inhabit the neighboring zones around Gangetic plain, the central zone of India was having only few cytotypes of *S. spontaneum* which consist of 2n=64 and its aneuploids. Chromosome number less than 64 was not reported from here. 2n=80 cytotype has been identified from all parts of the country except in North zone states. In many states it was in

low frequency whereas in Gujarat 80% of the *S. spontaneum* clones were with 2n=80. This disproves the prior assumption that this cytotype was restricted Assam and Western Ghats.

The world collection of sugarcane germplasm maintained by ICAR-Sugarcane Breeding Institute, Coimbatore, India is the largest germplasm collection at present. A large assembly of *S. spontaneum* representing the entire range of availability collected from its distributional areas of the country is currently available in the Institute. These accessions were used for the present study and it revealed that *S. spontaneum* is rich in genetic variability and are compatible group for inter and intraspecific hybridization. Polyploid series from lowest chromosome number 2n=5x=40 to the highest of 2n=14x=112 in this species has been revealed from this study. Natural hybridization between the cytotypes with multiples of 8 (x=8) resulted in other cytotypes and also aneuploids in its distributional areas. A total of 26 cytotypes were identified from India. In North and North-East India the evolutionary mechanism are highly active in this species and it is found that the cyto-morphological variability favor the accumulation of adaptability characters, especially to biotic and abiotic stresses. In sugarcane, compared to most of the other commercial crops, the information on genetic variability and geographic distribution pattern of the wild species, *S. spontaneum*, is available and it can be contributed to the development of superior clones with desirable characters.

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Effects of high temperature on mitotic index, microtubule and chromatin organization in rye (*Secale cereale* L.) root-tip cells

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Abstract. Stressful high temperatures on plants can limit whole-plant function and decrease crop productivity. However, little is known regarding heat stress effects on microtubule cytoskeleton and chromatin in roots from intact plants. Here we studied high temperature effects on cell division, microtubule and chromatin organization patterns in rye root tips from intact plants subjected to 40°C for 4 h and after different recovery periods (0RT, 7RT, 24 RT). We showed that heat stress induced changes in nuclear morphology as detected by the unusual presence of interphase cells with irregularly shaped nuclei, probably associated with changes in chromosome segregation at anaphase, leading to micronuclei formation as well as changes in the mitotic index. These alterations were associated to differential effects in microtubules organization in both heat-stressed interphase and mitotic cells at 0RT and 7RT. Although no changes in the distribution of H3 phosphorylation of Ser 10 residues on chromatin were found in cells from heat-stressed plants, marked alterations in chromatin DNA methylation patterns were detected. These effects included higher agglutination of 5-methylcytosine domains in both interphase and metaphase cells compared to controls. Taken together these results seem to suggest that alterations in microtubule conformation upon heat stress influences nuclear chromatin organization and cell cycle progression. However, when seedlings recovered from stress (24RT), root tip cells presented microtubule configurations and chromatin organization patterns similar to controls. We conclude that in spite of heat stress markedly altered cell cycle progression and distribution of epigenetic marks, these responses are transient to cope with such stress conditions in the roots.

Keywords: DNA methylation, heat stress, histone H3 Ser 10 phosphorylation, microtubules, root.

INTRODUCTION

High temperature is one of major environmental factors limiting crop growth and yield worldwide causing many physiological changes that affect crop yield and quality (Suzuki et al. 2014). Most studies on these effects focus on the above-ground tissues such as shoot and reproductive organs,

although roots can also be subjected to high temperature stress which can limit whole plant function and decrease productivity (Heckathorn et al. 2013). A high degree of complexity in plant responses at the molecular, physiological and biochemical levels were described, largely controlled by different, and sometimes opposing, signaling pathways that may interact and inhibit each other (Suzuki et al. 2014). However, knowledge concerning combined microtubule (MT) cytoskeleton organization and chromatin nuclear topology upon heat stress in intact plants is scarce, particularly in roots.

In the plant cell cycle, the MT cytoskeleton composed of heteropolymers of α- and β-tubulin undergo dynamic conformational changes in a process known as dynamic instability in response to the needs of the cell (Horio and Murata 2014). Particularly, during cell division in somatic cells, MTs are arranged into characteristic structures like the interphase cortical MTs (CMT), pre-prophase band, mitotic spindle and phragmoplast (Baluska et al. 1998). Moreover, MTs can undergo a number of posttranslational modifications that act to control specific MTs-based functions in plants, including tyrosination, detyrosination, acetylation (Smertenko et al. 1997a), phosphorylation (Blume et al. 2008), polyglutamylation (Wang et al. 2004), and transamidation (Del Duca et al. 2009).

In plant cells disruption of MTs occurs in response to various environmental factors namely to extreme temperatures such as heat stress (*Nicotiana tabacum*, Smertenko et al. 1997a; Smertenko et al. 1997b; *Arabidopsis thaliana*, Müller et al. 2007), low temperature and abscisic acid treatments (*Triticum aestivum*, Khokhlova et al. 2003), hyperosmostic stress (*Triticum turgidum*, Komis et al. 2002), and affects post-translational modifications of tubulin as in cadmium stress (*Glycine max*, Gzyl et al. 2015). Furthermore, plant MTs in addition to their role in cell division and axial cell expansion, also have a thermosensory function that is of agronomical relevance in osmotic or cold stress conditions (*Triticum aestivum*, Abdrakhamanova et al. 2003; Nick 2012).

Moreover, plants response to drought, cold and high salinity stress involve several epigenetic regulatory mechanisms like both DNA and histone methylation and generation of small RNAs implicated in genome regulation and structure (Mirouze and Paszkowski 2011; Asensi-Fabado et al. 2017). Recent research has shown that environmental cues and abiotic stresses activate a stress memory that is mediated by epigenetic and chromatin-based mechanisms including chromatin modifications, such as cytosine methylation of DNA, histone methylation and nucleosome occupancy (Lämke and Bäurle 2017). For example, exposure of *Arabidopsis* plants to stresses, including salt, UVC, cold, heat and flood, resulted in a higher homologous recombination frequency, increased global genome methylation, and higher tolerance to stress in the untreated progeny. However, this transgenerational effect did not persist in successive generations (Boyko et al. 2010)..By contrast, prolonged heat stress induces transcriptional activation of several repetitive elements of *Arabidopsis thaliana* that requires minor changes in histone modifications but does not involve DNA demethylation (Pecinka et al. 2010). Patterns of DNA and histone modification can be altered in root tip cells of soybean seedlings grown at different temperatures (Stępiński 2012). During male meiosis in *Secale cereale* plants with B and without B chromosomes, heat exposure causes differential anomalies in chromatin structure in pachytene cells (Pereira et al. 2017). Heat-damaged pachytene cells displayed easily recognizable paired chromosome fibres and a single amorphous and heterochromatic mass closely associated with the nuclear periphery as well as disruption of the organization of sub-telomeric chromosome regions. However, no changes in DNA methylation patterns were detected in untreated and treated plants (Pereira et al. 2017).

In this work we investigated the effects of heat stress (40°C 4 h) on the organization of MT arrays and chromatin in rye root tips from intact seedlings, immediately after stress (0RT) and at different recovery periods (7RT, 24RT). In-depth cytological analyses of chromatin and microtubular organization were performed in root tip cells with DAPI, and immuno-labelling with antibodies against tubulin, 5-methylcytosine (5-mC) and histone H3 phosphorylated at serine 10 residue (H_3S_{10}ph).

MATERIALS AND METHODS

Plant material and heat stress conditions

Rye (*Secale cereale* L., $2n = 14$ chromosomes) seeds were kindly supplied by Neil Jones (Aberystwyth, UK). Seeds were placed in Petri dishes with moistened filter paper in the dark at 4°C for 3 days. Seedlings were transferred to a growth chamber with controlled light-temperature (Rumed), with a photoperiod of 18 h light and 6 h dark, at 25°C ± 2°C for 2 days. Then, seedlings were subdivided in two sets of experiments: (i) kept at 25°C ± 2°C with a photoperiod of 18 h light and 6 h dark (controls); and (ii) exposed to a ramp of increasing temperature from 25°C to 40°C (temperature increases 2°C / h), remaining 4 h at 40°C, after which the temperature fell gradually to 25°C (temperature decreases 2°C / h). The heat stress temperature was chosen based

on agronomical relevant temperatures shown to have a significant effect in cool season grasses such as rye (Xu, Zhan et al. 2011). All seedlings were kept moist during heat stress. Root tips were collected from plants in control conditions and from treated plants immediately after exposure to heat stress 40°C (0RT), and 7 h (7RT) and 24 h (24RT) during stress recovery.

Immunolabelling

For immunostaining of α-tubulin root tips were prepared as described in Caperta et al. (2006). Briefly, roots were fixed in freshly prepared 4% paraformaldehyde solution (PFA) containing MT stabilizing buffer (1xMTSB) for 45 min at room temperature, and then rinsed twice in 1xMTSB for 5 min. For H_3S_{10}ph immunodetection, root tips were fixed in freshly prepared 4% PFA solution containing phosphate-buffered saline (1xPBS, pH 7.3) for 30 min, and then washed three times for 5 min in 1x PBS according to Caperta et al. (2008). Immunostaining of 5-mC was performed in root tips fixed in ethanol:acetic acid (3:1) as described in Carvalho et al. (2010). For both H_3S_{10}ph and 5-mC immunolabelling, root tips were digested by treating with a pectolytic enzyme mixture [2% cellulase (Sigma), 2% cellulase "Onozuka R-10" (Serva), and 2% pectinase enzyme (Sigma) solution in 1xEB at 37º] until the material became soft.

The macerated material was squashed in 1xPBS or 1xMTSB on a slide. Slides were incubated for 1 h at 37ºC in a moisture chamber with a blocking solution (3% bovine serum albumin (BSA) in 1x PBS/1xMTSB, 0.1% Tween 20), followed by an incubation at 10ºC overnight with the primary antibody diluted in 1xPBS/MTSB supplemented with 1% BSA. After three washes in 1xPBS/MTSB for 5 min, the secondary antibodies diluted in 1xPBS/MTSB supplemented with 1% BSA were applied for 45 min at 37ºC. After final washes with 1xPBS, slides were counterstained with 4'6-diamino-2-phenylindole (DAPI) and mounted in 1mg/ml Citifluor antifade medium (AF1, Agar Scientific).

For MTs immunolocalization a mouse monoclonal antibody to α-tubulin (clone DM1A, Sigma, 1:100) was used, and the tyrosinated form of α-tubulin was detected with a rat antibody (YOL 1/2, Serotec, 1:100). An anti-mouse Alexa 488 antibody (Molecular Probes) and an anti-rat antibody conjugated with biotin (Serotec, 1:200) were used as secondary antibodies. This latter antibody was further detected with a streptavidin-Cy3 conjugate antibody (Sigma, 1:700). For detection of H_3S_{10}ph, a rabbit antibody (Upstate, 1:200) was utilized and revealed using anti-rabbit rhodamine-conjugated antibody (Dianova, 1: 100). For revealing 5-mC a primary mouse antibody (Abcam, 1:200) and a secondary antibody anti-mouse-Cy3 (Sigma, 1:100) were used. After three final washes, the slides were counterstained with DAPI and mounted in 1mg/ml Citifluor antifade medium (AF1, Agar Scientific). All samples were examined using a Zeiss Axioskop 2 epifluorescence microscope, images were obtained using a Zeiss AxioCam digital camera, and the digital images were processed with Photoshop (Adobe Systems).

Quantitative analysis of cell cycle progression and MT organization

The nuclear morphology of well-preserved cells from control and in distinct recovery periods (0, 7 and 24 RT) after treatment was determined and the percentage of DAPI stained cells with either regular or irregular shaped nuclei, and cells with micronuclei were calculated. Cell cycle was evaluated by calculating the mitotic index as the percentage of mitotic cells identified in at least n = 200 cells for each treatment. The number of mitotic cells at different phases was moreover evaluated through tubulin immunolocalization of particular MTs configurations (e.g. preprophase band, mitotic spindle or phragmoplast) in both control and heat-stressed cells after distinct periods of stress recovery. Effects of heat stress on CMTs organization were also evaluated in 200 cells from each treatment through the quantification of cells with normal or new MTs arrangements in distinct recovery periods. The Chi-square test (χ^2, $P< 0.05$) was utilized for statistical analysis.

RESULTS AND DISCUSSION

Changes in nuclear morphology and in mitotic index after heat stress are associated with new, transient MT arrangements

Interphase cells were classified as normal, when nuclei present regular shape and well-defined contour; abnormal, those showing nuclei with irregular shape; and cells with micronuclei. Most control cells (n = 200) showed normal interphase nuclei (94%, Table 1; Fig. 1a,b) and the mitotic index was 6%, but decreased immediately after heat stress (0RT – 3%, n=302). In 0RT cells a significant difference in nuclei types was detected in comparison with controls ($\chi^2 = 35.31$, $P< 0.05$), with an increase in the frequency of cells with abnormal nuclei (21%) and cells with micronuclei (3%). At 7RT cells (n = 300) significant differences between

Table 1. Percentage (%) of interphase cells with nuclear normal morphology (DAPI), tubulin immunolabeled cells in interphase (CMTs) and mitosis, and mitotic index. Control and heat-stressed cells analyzed after 0 (0 RT), 7 (7 RT), and 24 (24 RT) h of recovery from the stress. The presence of cortical microtubules (CMT), preprophase band (PPB), mitotic spindle (SP) and phragmoplast (P) was scored.

Seedlings treatments	Frequencies (%) of DAPI stained interphase cells with normal nuclear topology	Mitotic Index	Frequencies (%) of interphase cells with typical organized CMTs arrays	Frequencies of mitotic cells (%) at distinct phases			Number of mitotic cells analysed
				PPB	SP	P	
Control	94[a]	6[a]	98[a]	64[a]	24[a]	12	33
0 RT	76[b]	3[b]	16[b]	78[b]	11[b]	11	37
7 RT	53[c]	18[c]	37[c]	48[c]	27[a]	25	48
24 RT	90[a]	9[d]	95[a]	33[d]	48[c]	19	60

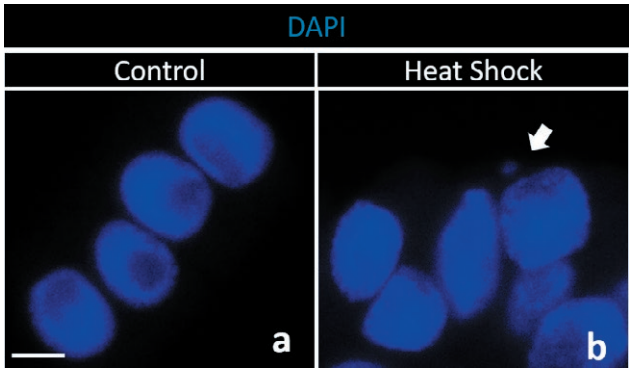

Figure 1. DAPI-stained *Secale cereale* meristematic interphase root cells. 1a – Control cells with a well-defined contour; and 1b. heat-stressed cells with irregular nuclei and/or with micronuclei (arrowed). Bar: 5 μm.

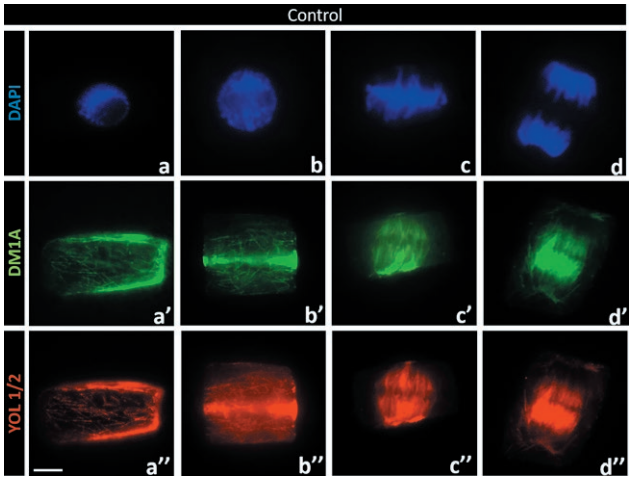

Figure 2. Indirect immunodetection of α-tubulin (MT) in control cells. Nuclei, chromatin and chromosomes are stained with DAPI (blue). Tubulin containing arrays are detected with DM1A α-tubulin (green) and YOL1/2 tyrosinated α-tubulin (red) antibodies. **a- a"** Interphase cell with cortical microtubules; **b- -b"** prophase cell with the preprophase band; **c -c"** meta/anaphase cell with spindle; **d- d"** ana/telophase cell with the phragmoplast. Bar: 5 μm.

heat-stressed cells and controls were found ($\chi^2 = 92.36$, $P< 0.05$) with a frequency of abnormal nuclei more than doubled (41%), and a small increase in cells with micronuclei (6%). The observed increase of micronuclei frequency attributable to root tips heat exposure is moreover associated with heat stress effects detected on mitotic cell cycle progression. The mitotic index was three times higher at 7RT (18%, Table 1) than in controls. Contrastingly, 24RT cells (n = 275) presented a high frequency of normal nuclei (90%) and a decrease in mitotic index (9%). The frequency of abnormal nuclei (7%) and micronuclei markedly decreased (3%). These findings support the hypothesis of mitotic arrest after 7RT of exposure to heat stress. Root tips exposure to diverse chemical substances including fertilizers, heavy metals, herbicides, pesticides and radioactivity also affect the mitotic index in varying frequencies in *Allium cepa* (Bonciu et al. 2018). Nonetheless, contrasting effects in the mitotic index were also observed in *Secale cereale* plants exposed to chemical stresses like the MTs-depolymerizing agent colchicine where mitotic arrest occurred in the low-concentration treatment, whereas c-metaphase cells were able to progress into the cell cycle in the high-concentration treatment (Caperta et al. 2006). Heat stress effects in nuclear morphology can also result from perturbations of CMTs organization as previously described for colchicine treatments (Caperta et al. 2006).

MTs configurations were analyzed in untreated and heat-treated root tips through tubulin immunolocalization using antibodies that recognize α-tubulin (DM1A) and tyrosinated tubulin (YOL 1/2). Our results show that both antibodies presented coincident and similar immuno-signal distributions (Fig. 2). Control cell MTs exhibited both α-tubulin and tyrosinated tubulin arrays

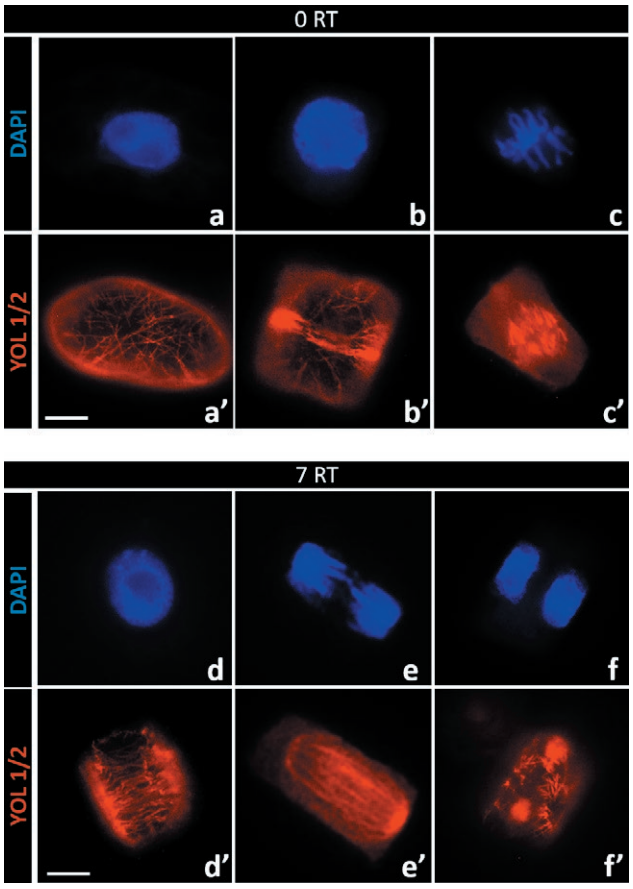

Figure 3. Indirect immunodetection of α-tubulin (MT) in heat-stressed cells after 0 (0 RT), 7 (7 RT), and 24 (24 RT) h of recovery from the stress. Nuclei, chromatin and chromosomes are stained with DAPI (blue). Tubulin containing arrays are detected with YOL1/2 tyrosinated α-tubulin (red) antibody. **a-a'** and **d-d'** Interphase cells showing branched, stringy CMTs with disorganized orientation; **b-b'** prophase cell with a slightly disorganized pre-prophase band with MTs without the usual parallel orientation; metaphase cell (**c-c'**) and anaphase cell (**e-e'**) with normal spindles; **f-f'** telophase cell with thick CMTs arrays and with remnants of phragmoplast. Bar: 5 μm.

configurations characteristic of higher plant cells: CMTs at interphase, pre-prophase band, mitotic spindle and phragmoplast at the end of telophase (Fig. 1). Control cells presented organized CMTs (98%, Table 1), whereas at 0RT significant differences occurred (χ^2 = 687. 57, P< 0.05), in which the majority of cells exhibited branched and wavy CMTs with a disorganized orientation (84%) (Fig. 3a-a'). Previous studies showed that heat stress caused dissemblance of MTs in mitotic tobacco suspension cultured cells after 30 min at 42°C (Smertenko et al. 1997b). In the present study we showed higher resilience of rye MTs to heat stress since exposure of 2 days seedlings to 4 h at 40°C only induced CMTs disorganization at 0RT without total disruption. However, knowledge is inexistent with regard to the heat response of the rye variety used in this study (heat-resistant or heat-sensitive). Compared to controls, at 7RT there was still a significant difference in the frequency of interphase cells with a disorganized and branched wavy-like MTs configuration (63%, Table 1, Fig. 3d-d') (χ^2 = 424. 97, P< 0.05), although the frequency of interphase cells with normal MTs arrangements doubled. At 24RT 95% of interphase cells present typical organized CMT arrays (Table 1). Disorganized wavy MTs arrangements were also observed in root cells exposed to high colchicine concentration conditions (Lazareva et al. 2003; Caperta et al. 2006). Therefore, it is tempting to suggest that new MTs re-orientations result from perturbations induced by heat stress on CMTs associations with plasma membrane, although the nature of MT attachments to the membrane is not yet totally clear both in animal (Wolff 2009) and in plant cells despite all efforts to understand it (Liu et al. 2015). It was demonstrated that CMTs can change their orientation in response to a broad range of abiotic signals (Nick 2013) by controlling the direction of cellulose deposition and reinforcing axial cell expansion (Geitmann and Ortega 2009). In the current work, the high frequency of interphase cells with abnormal nuclear morphology observed at 7RT probably reflects changes in CMTs organization by inducing differential cytosol compartmentalization.

Earlier studies moreover reported that different MTs arrays presented distinct sensitivities to temperature stress (Smertenko et al. 1997b; Abdrakhamanova et al. 2003; Müller et al. 2007). The most heat-sensitive MT arrays are those of the mitotic spindle and the phragmoplast in tobacco cultured cells (Smertenko et al. 1997b). In *Triticum* chilling sensitive species, CMTs are extremely cold-sensitive, whereas they persist at low temperatures in chilling-tolerant species (Abdrakhamanova et al. 2003). In this study, MTs from the pre-prophase band (PPB) appeared to be very sensitive to heat stress since most prophase cells at 0RT (94%) presented slightly disorganized pre-prophase bands (Fig. 3b-b'). Such marked changes in frequencies of prophase cells with abnormal PPB at 0RT were however not associated with perturbations in other mitotic phases since all metaphase (Fig. 3c-3c') and anaphase cells exhibited well-formed spindles, and telophase cells showed phragmoplast MTs orthogonally disposed to the division plane. After 7RT, most prophase cells presented a reduction of abnormal pre-prophase bands (65%), although in some cells altered spindle and phragmoplast configurations were revealed (Fig. 3f-f'), which were absent at 24RT.

Compared to controls, the frequency of cells with PPBs presented the highest value at 0RT (78%) and the

lowest one at 24RT (33%). These findings contrasted with frequencies of cells with spindles and phragmoplasts, which have low frequencies at 0RT (22%) and high frequencies at 7RT (52%), with a maximum of at 24RT (67%). Taken together, the drastic dropping of mitotic index values at 0RT seems to be associated with perturbations on CMTs organization as well as disturbances on PPB allowing however the progression of subsequent mitotic phases as only cells with normal spindles and phragmoplasts were detected. On the other hand, at 7RT the observed beginning of normal CMT organization reestablishment seemed to allow cells transition to mitosis associated with the marked increase in the mitotic index. The high frequencies of cells in metaphase, anaphase and cytokinesis at 7RT, associated with some perturbations in spindle and phragmoplast organizations appear to indicate why heat-stressed cells stay longer in mitosis. After 24 RT the MT cytoskeleton configurations in interphase and prophase cells were like the ones observed in controls, although the high frequencies of cells at metaphase, anaphase and cytokinesis revealed a delayed reestablishment reflected in the high mitotic index yet observed. Nonetheless, it is not yet clear which microtubule structures, cortical MTs or mitotic MTs, are susceptible to tubulin modification induced by heat stress.

Heat stress induces changes in DNA methylation patterns but no alterations in $H_3S_{10}ph$ distribution patterns

In both control and heat-stressed cells $H_3S_{10}ph$ marks were absent during interphase. However, they were present in prophase cells in the pericentromeric heterochromatin and restricted to one nuclear hemisphere revealing Rabl configuration (Fig. 4). The Rabl configuration, characteristic of rye genome (Caperta et al. 2002) is maintained after heat stress as centromeres are all aligned in one nuclear pole. In interphase cells no labelling was found (Fig. 4a-a'), which contrasted with prophase (Fig. 4b-b'), metaphase (Fig. 4a-a' and c-c') and anaphase (Fig. 4a-a') cells. In mitotic cells, a marked labelling was also found in chromosomes pericentromeric regions but no detectable changes were observed in signal dimensions or intensities in both control and heat-stressed cells (Figs. 4d-d', 4e-e', 4f-f'). Instead, cold treatment of plant root meristems resulted in additional chromosomal sites of $H_3S_{10}ph$, besides the usual ones in pericentromeric regions (Manzanero et al. 2000). Also, up-regulation of the stress-inducible genes in *Arabidopsis* T87 and tobacco BY-2 cell lines is associated with increased phosphorylation of histone H3 at serine 10 residue at high salinity, cold and abscisic acid treatments (Sokol et al. 2007).

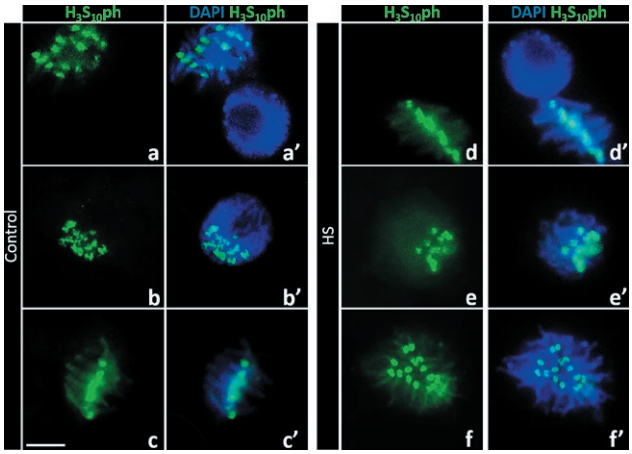

Figure 4. Indirect immunodetection of histone H3 phosphorylated at serine 10 residue in *Secale cereale* meristematic root cells. Nuclei, chromatin and chromosomes are stained with DAPI (blue). Chromatin and chromosomes are detected with histone H3 phosphorylated at serine 10 residue ($H_3S_{10}ph$, green) antibody in control (a-a'- c-c') and heat-stressed cells (d-d'- f-f'). **a-a'** Interphase cell without $H_3S_{10}ph$ labelling and an anaphase cell with $H_3S_{10}ph$ marks; **b-b'** prophase cells showing $H_3S_{10}ph$ marks in the pericentromeric heterochromatin and restricted to one nuclear hemisphere revealing Rabl configuration; **c-c'** metaphase cell presenting $H_3S_{10}ph$ dots only in a discrete region in pericentromeric chromatin; **d-d'** cell at interphase showing no $H_3S_{10}ph$ labelling and a metaphase cell with pericentromeric $H_3S_{10}ph$ immnosignals; **e-e'** cell at prophase with $H_3S_{10}ph$ marks in the pericentromeric heterochromatin; and **f-f'** early anaphase cell revealing $H_3S_{10}ph$ dots only in pericentromeric chromatin. Bar: 5 μm.

Control cells showed interphase nuclei with disperse, dotted and intense 5-mC immunosignal all over the nucleus both in highly condensed (heterochromatin) and decondensed (euchromatin) chromatin (Fig. 5a-a''). Instead, in heat-stressed cells at 0RT and 7RT a distinct, heterogeneous DNA methylation distribution pattern with aggregated immunosignal was preferentially found more concentrated in the nuclear periphery (Fig. 5c-c'' and 5d-d''). In metaphase cells, at both 0RT and 7RT a heterogeneous and discontinuous 5-mC labeling was also detected along chromosome arms (Fig. 5e-e''). After 24 RT, the distribution patterns of 5-mC were like those observed in control interphase and metaphase cells (Fig. 5f-f'''). The results presented here in rye somatic cells from control plants are in accordance with earlier studies in rye metaphase chromosome spreads, which displayed a punctuated and uniform pattern of methylated DNA residues along both the As and Bs chromosomes, without any particular sites of accumulation (Carchilan et al. 2007). However, no differences were found in the nuclear distribution of methylated cytosines between meiocytes of heat-stressed and control rye plants with

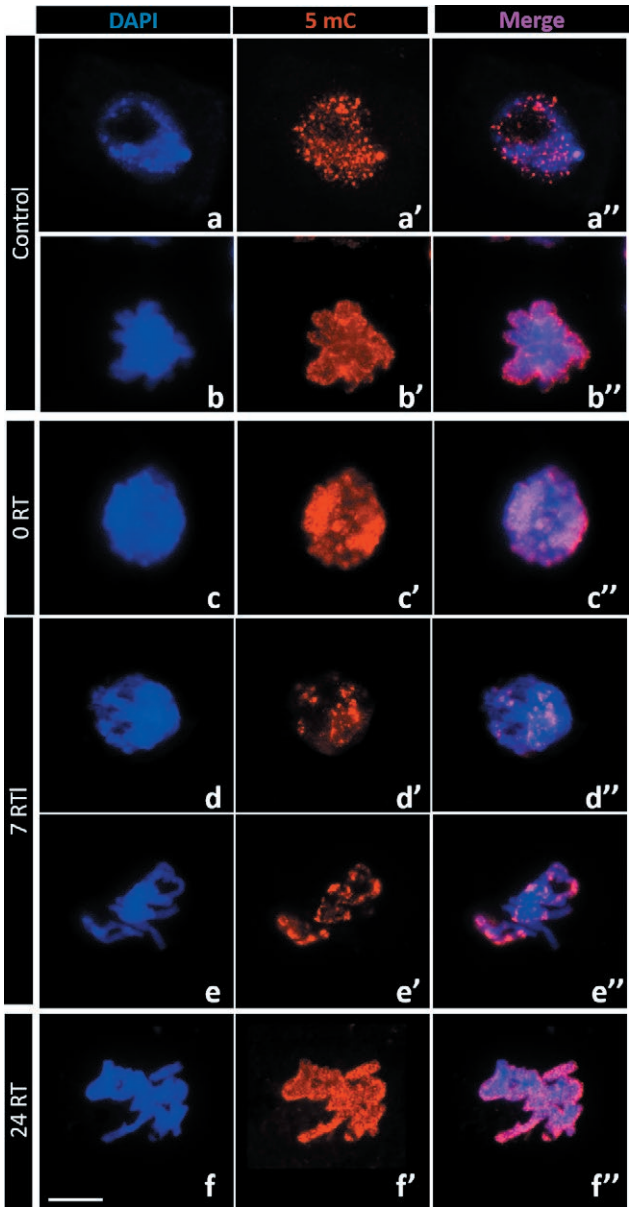

Figure 5. Indirect immunodetection of 5-methylcytosine in *Secale cereale* meristematic root cells. Control and heat-stressed cells were analysed after 0 (0 RT), 7 (7 RT), and 24 (24 RT) h of recovery from the stress. Nuclei, chromatin and chromosomes are stained with DAPI (blue). Chromatin and chromosomes are detected with 5-methylcytosine (5-mC, red) antibody. **a-a"** Control interphase and **b-b"** metaphase cells with homogenous 5-mC labelling; **c-c"** and **d-d"** heat-stressed interphase nuclei with a heterogeneous distribution pattern of 5-mC exhibiting aggregated immunosignals in the nuclear periphery; **e-e"** an heterogeneous discontinuous 5-mC labelling along chromosome arms of a metaphase cell; **f-f'"**metaphase cell showing a homogenous 5-mC labelling after 24RT. Bar: 5 μm.

0B or 2B chromosomes (Pereira et al. 2017). A study on the effects of heat stress in the repetitive sequence genome fraction (coding and non-coding sequences) using leaves of intact rye plants suggests marked differences between these sequences that most likely reflect their distinct roles in the plant pathways involved in the stress response (Tomás et al. 2013).

Hence, it appears that there are different temperature chromatin sensitivities accordingly with chromatin fractions, cell types and tissue origins. For instance, in soybean at normal temperature, root hairs were more hypermethylated than were stripped roots whereas in response to heat stress, both root hairs and stripped roots showed hypomethylation (Hossain et al. 2017). These changes in DNA methylation were directly or indirectly associated with expression of genes and transposons within the context of either specific tissues/cells or heat stress (Hossain et al. 2017). Other studies in which both roots and above-ground tissues were heat-stressed indicate that roots are often more sensitive to heat stress than shoots in terms of cell division and physiological responses (Heckathorn al. 2013). We conclude that heat stress induces transient changes in chromatin and MT organization rye root seedlings, which might indicate cell adjustments in stressful conditions.

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Investigation of the Cytotoxic and Genotoxic Effects of the *Euphorbia rigida* Bieb. Extract[1]

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Abstract. The present study was conducted to evaluate and compare the cytotoxic and genotoxic effects of the aqueous extracts of *Euphorbia rigida* Bieb. which is a natural pesticide. The comparison was done using the *Allium* test to the chemical pesticides Elandor® and Goldplan®. According to *Allium* test results, it had negative impacts on mitosis and showed cytotoxic and genotoxic effects on the existing cells. The lowest level of MI (2.95 %) was observed in the 200 ppm treatment of *E. rigida* extract. Number of the aberrant cells were 88.1, 84.1 and 82.5 in the treatments with Elandor®, 50 ppm, *E. rigida* extract and Goldplan® respectively. The highest cytological anomalies were chromosome stickiness, irregular metaphase and anaphase, pole deviations and C-mitosis. According to the present results of this research, we can suggest that the extract obtained from *E. rigida* plant with water up to 50 ppm can be used as an alternative to chemicals used as biopesticide. Antibacterial, antifungal and antiviral properties of *Euphorbia* extracts are well known, so it could mean that they can be used as additives or a disinfectant for inanimate surfaces in the pharmaceutics industry. No matter what purposes the extract of this plant is used, great care should be taken while using it because it can cause damage on cells and chromosomes. Hence, through more detailed and comprehensive studies about its capacity for medical and biopesticide purposes should be investigated.

Keywords: *Allium* test, Biopesticide, Cell aberrations, Cytotoxic, Genotoxic and *Euphorbia rigida* Bieb.

INTRODUCTION

Extracts and essential oils of some naturally grown plants show antibacterial and antifungal activities and these activities are used as a biopesticide in agricultural control and nutrient preservation (Baytop 1991). In recent years, researchers have been focusing on studies to obtain compounds harmless to human health and the environment that can be used instead of chemical pesticides against plant diseases and pests, which are of great economic value. Although pesticides have a fast and strong effect in the con-

trol of pests, they cause environmental pollution and accumulate over time in all living things through the food chain, creating toxic hazard (Güler and Çobanoğlu 1997).

Most of the *Euphorbia* species are rich in phenolic compounds, aromatic esters, diterpenoids, tetracyclic and pentacyclic triterpenoids, essential oils, resins, resinoids and many bioactive compounds (Wu et al. 2009; Kumar et al. 2010; Ekeke and Ndukwu 2014; Ghareeb et al. 2018; Ghosh et al. 2019). Diterpenoids of *Euphorbia* have biological activities such as antitumor, cytotoxic, anti-viral and anti-inflammatory, but flavonoids and tannins are known for their antitumor, hepatoprotective and antioxidant activities (Wu et. al. 2009; Ghareeb et al. 2018; Ghosh et al. 2019). The plants of *Euphorbia* species are used for the treatment of hypertension, destruction of wart cures, skin diseases, gonorrhoea, migraine and intestinal parasites (Kırbağ et al. 2013; Ghareeb et al. 2018). *E. hirta* possesses antibacterial, anthelmintic, antiasthmatic, sedative, antispasmodic, antifertility, antifungal, and antimalarial properties (Kumar et al. 2010; Özbilgin and Çitoğlu-Saltan 2012). Compounds isolated from *E. paralias* showed moderate antiviral activity against HIV-1 replication. Seven triterpenoids isolated from *E. antiquorum* and steroids isolated from *E. chamaesyce* also have strong inhibitory activity against Epstein-Barr virus early antigen (EBV-EA) activation (Shi et al. 2008). *E. paralias, E. maschallian* and *E. myrsinites* species have different diterpene species that have been identified as antiviral compounds. Some of the other *Euphorbiaceae* species, e.g. *E. pekinensis, E. peplus*, *Phyllanthus nanus* and *P. amarus* are effective against virus infections (Gyurıs et al. 2009). The aqueous and 50% MeOH extracts of *E. hirta* shows direct antiviral effects on HIV-1, HIV-2 and SIV (mac251) reverse transcriptase (RT) activity which were determined in MT4 cells *in-vitro* (Gyurıs et al. 2009; Alam et al. 2016). The antibacterial effect of *E. hirta* (Linn.) comes from tannins, alkaloids and flavonoids contained in the ethanol extract (Ogueke et al. 2007). *E. orientalis* L. was found containing bioactive compounds that has essential antibacterial and antioxidant activities (Avcı et al. 2013). The aqueous extract of *E. hirta* also inhibits aflatoxin contamination in rice, wheat, maize, and mustard crops (Kumar et al. 2010). *E. platypyllos* L. extracts showed significant cytotoxic effect and DNA damaging effects in MCF-7 cells (Aslantürk and Aşkın-Çelik 2013). The active ingredients of 17-Acetoxyjolkinolide B and 13-hexadecanoyloxy-12-deoxyphorbol were obtained from the roots of *E. fischeriana*. Cytotoxic effects of these compounds against Ramos B cells were already determined (Wang et al. 2006; Özbilgin and Çitoğlu-Saltan 2012). Dafnan and tigliane diterpenoids isolated from latex of *E. poisonii* showed selective and potent cytotoxic effects on human kidney carcinoma (A-498) cell lines (Wang et al. 2006; Shi et al. 2008). Ingenol 3-angelate that is *Euphorbia* diterpenes, which was approved by the FDA in 2012 and the EMA in 2013 for the treatment of actinic keratosis, a precancerous skin condition. *E. retusa* extract was effective for the prevention of CCl_4-induced hepatic damage in rats (Ghareeb et al. 2018). Extracts of *E. hirta* have been found to show anticancer activity (Kumar et al. 2010). The latex and plant extracts derived from the roots of the *E. rigida* plant did not cause gene change in bacteria in two sensitive Ames test strains, such as TA 98 and TA 100, while in the Comet experiment, they showed mutagenic effects in human lymphocytes (Başaran et al. 1996). Fumigant effect of *E. aleppica* extract has been demonstrated with an average of 88% mortality against grain storage pests, *Sitophilus garanarius* and *S. oryzae* (Şahin et al. 2006). The strong molluscoidal activity of water extracts and partially purified latex of *E. pulcherima* and *E. hirta* plants is known (Shi et al. 2008). Isolated compounds from *E. paralias* had strong molluscicidal activity on *Biomphalaria alexandrina* (Ehrenberg) and antifeedant effects on third-instar larvae of *Spodoptera littoralis* (Boisd) (Abdelgalil 2002). The petroleum ether fraction of *E. hirta* is indicated as an herbicidal biopesticide with a larvicidal effect in *Anopheles stephensi* known as the malaria vectors (Huang et al. 2012). Methanolic extract of aerial parts of *E. hirta* was effective against *P. falciparum* parasites, polyphenolic extract of *E. hirta* inhibited the growth of *Entamoeba histolytica (*Kumar et al. 2010) and aqueous leaf extracts (1:10 w/v) of *E. hirta* had a maximum killing efficacy (45%) against the *Zonabris pustulata* Thunb. (Oudhia 2000).

E. fischeriana has been used as an anthelmintic and insecticide in China (Lee et al. 1991). The aqueous extract of *E. hirta* reduced the egg counts of intestinal parasites in Nigerian dogs' feces as a potential anthelmintic and antiparasiticide agent (Huang et al. 2012). The ethanol extracts from the leaves and flowers of *E. cyparissias* L., had the highest larvacidal activity against the codling moth (*Cydia pomonella* L.) with strong acaricidal activity on the treated population of two-spotted spider mite (*Tetranychus urticae* Koch.) on the third day post treatment (Velcheva 2001). Extracts of *E. hirta* and the root exudate exhibit nematicidal activity against juveniles of *Meloidogyne incognita* (Kumar et al. 2010). *E. myrsinites* extracts against root-knot nematodes (Nematoda: Meloidogyne spp.) in greenhouse tomato cultivation were found to be significantly more effective than synthetic pesticide Cyromazine ($C_6H_{10}N_6$) (Civelek

and Weintraub 2004). Most plant extracts have been used as topical antiseptics, or have been reported to have antimicrobial properties (Avcı et al. 2013).

Most of the *Euphorbia* species and other plant extracts, which are used for some medicinal and agricultural purposes, show potentially toxic, mutagenic, carcinogenic and teratogenic effects. For this reason, it is necessary to test the potential harmful effects of plant extracts to be used in both medical (antibacterial, antifungal, antiviral, antitumor, antioxidant, antiseptic, anthelmintic etc.,) and agricultural purposes (biopesticide, insecticidal, acaricidal, antiparasitic, larvicidal, molluscoidal, antimalarial, antifeedant etc.,). In the current study, the cytotoxic and genotoxic effects of *E. rigida* (Bieb.) being used for medical and biopesticide purposes were investigated. The objective of this study was to evaluate and compare the cytotoxic and genotoxic effect of the extract of *E. rigida*, a natural pesticide, with the chemical pesticides, Elandor® and Goldplan®.

MATERIALS AND METHODS

Collection of the plants and their extraction

The aerial parts of *Euphorbia rigida* (Bieb.) in the flowering period were collected from Sıtkı Koçman University campus (GPS:37°09'40,1"N 28°22'34"E) in Muğla at Turkey. The taxanomic identification of plant materials was confirmed and deposited in the herbarium by voucher specimen Dr. Olcay Ceylan at the Department of Biology at Muğla Sıtkı Koçman University (Herbarium number: O0388). The samples were air-dried at room temperature and protected from direct sunlight. Seventyfive (75 g) grams of dried and powdered aerial parts of *E. rigida* were extracted with 2.5 L boiling water for 60 min. Decoction (aqueous phase) was filtered with a 2.5 µm filter paper (Whatman No. 42) to remove suspended particles and the extract was kept at least 3 days at -20°C and later lyophilized to obtained crude (6.72 g) extract which was stored at -20°C.

Allium test method

The United Nations Environment Program (UNEP) and the US Environmental Protection Agency (USEPA) have standardized the use of plants as bioindicators in the determination of toxicity (Sivas and Gökbayrak 2011; Girasun et al. 2019). UNEP and the International Chemical Safety Program (IPCS) certified the *Allium cepa* (onion) root tip test in 1991 as a highly effective biotester for imaging mutagenic effects (Oney-Birol and Gündüz 2019). The onion genotoxicity test provides for easy screening of chemicals or toxic agents with genotoxic, cytotoxic, physiologic, clastogenic and aneugenic effects, especially to plants. The *A. cepa* assay is an efficient test for chemical screening and *in situ* monitoring for genotoxicity of environmental contaminants (Sivas and Gökbayrak 2011; Pandey et al. 2014; Girasun et al. 2019; Adhikari 2019). The test has been used widely to study genotoxicity of many pesticides revealing that these compounds can induce chromosomal aberrations in root meristems of *A. cepa*. The most important advantage of this test is that it is a low budget method, which besides being fast and easy to handle, it also yields reliable results (Fiskesjö 1985, Rank 2003; Çelik and Aslantürk 2006; Eren et al. 2017; Karaismailoğlu 2016; Adhikari 2019; Liman et al. 2020). *Allium* test results show a good correlation with the other eukaryotic and prokaryotic test results (Bonciu et al. 2018; Pirdal and Liman 2019; Oney-Birol and Gündüz 2019; Adhikari 2019).

The effects of the extract on cells and chromosomes were investigated through *Allium* test (Fiskesjö 1981). For the *Allium* test, *A. cepa* were purchased from the market. 0, 50, 100, 200 and 400 ppm (v/v) solutions of *E. rigida* extract were prepared with distilled water and also 200 ppm Elandor® (Imidacloprid) and 400 ppm Goldplan® (Acetamiprid) solution (v/v) (recommended doses) was prepared. Elandor® and Goldplan® are commercially available pesticides, which are contact and systemic insecticide for the control of *Hemiptera*, especially aphids, *Thysanoptera* and *Lepidoptera* (Öncüer 2004).

All onions were grown in distilled water for first 48 hours. Then *A. cepa* bulbs were placed in beakers including 0 (control= distilled water), 50, 100, 200 and 400 ppm (v/v) solutions of *E. rigida*, 200 ppm Elandor® and 400 ppm Goldplan® (v/v) solution (for the treatment 24 h). At the end of 24 h (after total 72 h), the root number and length of *A. cepa* were determined. Also the least ten root tips from each treatment were fixed in Carnoy solution (absolute alcohol: chloroform: glacial acetic acid, 6:3:1). Thereafter, roots tips were applied to cold hydrolysis and the meristem tissue cells were painted with Feulgen, then squashed and examined under the light microscope (Nikon UFX-2A) (Elçi 1994). From these squashed root tips, ten random areas were selected for the observation at 10X40 microscopic magnification (approximately 2000 cells were counted in an area), minimum 20.000 mitotic cells were counted from each of the slides. On each slide, abnormalities of chromosome stickiness, binucleate cells, micronuclei, C-mitosis, lagging chromosome, fragmentation in the metaphase, bridges and pole deviations in anaphase, irregular metaphase or anaphase etc. were detected. Moreover, the

mitotic index (MI) was calculated for each treatment as a number of dividing cells/100 cells (Metin and Bürün 2008).

Statistical analysis

Statistical analyses were performed using the SPSS 14.0 Software Package Program, data were evaluated with One-Way ANOVA and LSD (Least Significant Difference) tests.

RESULTS AND DISCUSSION

The effects of the extract of *E. rigida*, a natural pesticide were compared with Elandor® and Goldplan®, chemical pesticides. Aqueous extracts of *E. rigida* plants (50, 100, 200 and 400 ppm), 200 ppm Elandor® and 400 ppm Goldplan® were observed to cause the occurrence of aberrant cells and considerably decrease cell division depending on the increase in extract concentration.

The number and length of root according to treatments

The roots of all onions left for rooting in distilled water for 48 hours were healthy and well developed, and the differences were observed in the average root length and the total number of roots due to the onion's own characteristics. In the 24 hours following the first 48 hours, the development of the roots of onions exposed to different doses of *E. rigida* extracts and chemical pesticides slowed down, the number of roots did not change and the root lengths were in different statistical groups ($p<0.05$) (Table 1). Changes in root length of the treatments were compared to the control at the inhibition percent. Growth in onion root apical meristems of the treatment samples was 45% less than that of the control. Accordingly, the extracts and chemicals applied most likely contain toxic substances with a sublethal effect ($p<0.05$). Inhibition of root growth could be not only related to apical meristematic activity but also cell elongation during differentiation or enzyme activation that promote the elongation and loosening of the cell wall in the differentiation process (Eren et al. 2017; Pirdal and Liman 2019).

The lowest root length (highest inhibition) in 24-hour treatment of extract and chemicals was observed in 400 ppm *E. rigida* treatment ($p<0.05$) (Table 1). The root elongation % inhibition of Elandor® and Goldplan® treatments remained below the 50 ppm dose of the extract ($p<0.05$). Root elongation inhibition increased with concentration increase of plant extract.

This means that the plant extract prevents mitosis by showing toxic effects on the meristematic cells of *A. cepa*. Heavy metal induced toxicity and mutagenicity on various plant species have been already reported. Primary toxic effect of *Pb* in higher plants had been the inhibition of root growth possibly due to the inhibition of cell division in the root tip region. The reduction in root lengthening is strongly correlated to the mitotic index of the root tips of *Lathyrus sativus*. The reductions in the number of mitotic cells in root tips of seeds exposed to *Pb* could be due to its mechanism of action on cell cycle progression (Adhikari 2019).

Effect on the mitotic index (MI) of treatments

Mitotic index (MI) is a cytogenetic parameter that helps to measure the proliferation (M phase) of mitotic cells (Oney-Birol and Gündüz 2019). After applications, the highest level of MI (15.70 %) was observed in the Elandor® treatment, and this was followed by the Goldplan®, 50 ppm, *E. rigida* extract and control. The lowest level of MI (2.95 %) was observed in the 200 ppm treatment of *E. rigida* extract ($p<0.05$) (Table 2). *A. cepa* meristem cells are not affected by the level of toxicity of these chemical pesticide solutions. In contrast, some chemicals involved in Elandor® and Goldplan® or the lowest plant extracts (*E. rigida* 50 ppm) may promote the cells into mitosis. However, by increasing the treatment doses of plant extracts, the toxic impact prevents cell division, and, as a result MI decreases ($p<0.05$) (Table 2). The decline in MI value shows interference in the cell cycle (Oloyede et al. 2009). The reduction in number of the dividing cells in the roots shows the cytotoxic effects of the substances that are found in the plant aqueous extracts. MI, number of aberrant cells and its percent were high in the control and *A. cepa* meristematic tissue cells which were exposed to *E. rigida* 50 ppm, Elandor and Goldplan solutions. This indicates that at the end of 24-hour exposure, treatment doses have enough influence on stopping the mitosis except the *E. rigida* 200 ppm ($p<0.05$). On the other hand, in *E. rigida* 100 ppm and *E. rigida* 400 ppm treatments, the defence systems preventing mitosis become active and this results in the decrease of MI. The high MI exposed to Elandor, Goldplan and 50 ppm *E. rigida* extract indicates that the damage on living cells from these extracts can be recoverable and tolerable. Significant reduction in MI may be due to disturbed cell cycle such as blockage of G1 phase and suppressing DNA synthesis or inhibition of DNA synthesis at the S phase or blocking of G2 phase preventing the cell from entering in mitosis or mitotic phase changes (Pandey et al. 2014; Karaismailoğlu 2017a;

Table 1. Mean root length (mm) and number of *Allium cepa* before and after the treatment with the different doses of *E. rigida* extract and the chemical pesticides.

First 48 h (Before the treatment)		After the treatments (After 72 hours)				
At the end of 48 h Root length (mean) (mm)± SD	Treatments and Doses (ppm)	At the end of 72 h Root length (mean) (mm)± SD	Increase in root length (mm ±SD)	Increase in percent of root length (%)		Root number ± SD
				% Growing	% İnhibition	
19.00 ± 5.25 d	Control (0)	22.00 ± 5.31 e	3.00	100	0	71 f
14.60 ± 4.70 c	*E. rigida* (50)	16.10 ± 4.64 c	1.50	50	50	30 a
19.34 ± 6.08 d	*E. rigida* (100)	20.23 ± 6.12 de	0.89	29.6	70.4	47 e
9.48 ± 2.51 a	*E. rigida* (200)	10.33 ± 2.43 a	0.85	28.3	71.7	33 c
12.40 ± 4.33 b	*E. rigida* (400)	13.00 ± 4.32 b	0.60	20	80	37 d
10.73 ± 2.58 ab	Goldplan (400)	12.76 ± 3.14 b	2.03	67.6	32.4	30 a
17.62 ± 4.96 d	Elandor (200)	19.34 ± 5.02 d	1.72	57.3	42.7	32 b

Variability around the mean was represented as ± SD (Standart Deviation). Data having the same letter in a column were not significantly differed by LSD's multipli comparison test (P<0.05).

Table 2. The number of normal, total normal and total aberrant dividing cells and percentage of total aberrant dividing cells in mitotic phases and mitotic index (MI %) for the chemical pesticides and the different treatment doses of *E. rigida* extract.

Treatments and Doses (ppm)	Prophase ± SD	Metaphase ± SD	Anaphase ± SD	Telophase ± SD	The number of total normally dividing cells ± SD	Total Aberrant Cell number ± SD	% Aberrant Cells ± SD	Total dividing cell number ± SD	% MI (Mean ±SD)
Control (0)	81 ± 11.97 c	61 ± 10.08 c	28.2 ± 6.95 c	42.9 ± 11.60 c	213.1	60.7	22.16	273.8	13.69 d
E. rigida (50)	82 ± 18.13 c	57.8 ± 18.89 c	30.3 ± 9.26 cd	31.7 ± 7.70 b	201.8	84.1	29.41	285.9	14.29 d
E. rigida (100)	59 ± 11.97 b	41.3 ± 10.39 b	17.9 ± 7.74 b	23.8 ± 9.02 b	142	48.5	25.45	190.5	9.52 c
E. rigida (200)	18.2 ± 6.12 a	9.6 ± 3.83 a	6.3 ± 2.54 a	7.3 ± 2.35 a	41.4	17.6	29.83	59	2.95 a
E. rigida (400)	56.1 ± 8.99 b	39.9 ± 10.99 b	18 ± 4.26 b	24.4 ± 6.61 b	138.4	46.5	25.14	184.9	9.24 b
Goldplan (400)	90.3 ± 14.29 c	55.2 ± 20.38 c	36.7 ± 11.47 d	46.3 ± 12.32 c	228.5	82.5	26.52	311	15.55 e
Elandor (200)	88.5 ± 8.51 c	57.3 ± 18.01 c	37 ± 8.89 d	43.2 ± 11.69 c	226	88.1	28.04	314.1	15.70 e

Variability around the mean was represented as ± SD (Standart Deviation). Data having the same letter in a column were not significantly differed by LSD's multipli comparison test (P<0.05).

Liman et al. 2020). The causes of the decrease in the MI can be physiological response of cells that have entered the mitotic cycle and are not protected against extract components; partial inhibition of energy, protein, RNA and DNA synthesis in treatment groups and inhibition or postponement of the mitotic spindle formation in treated groups due to the high percentage of prophase in some concentrations (Karaismailoğlu 2016).

Formation aberrant cells according to treatments

Increase in the frequency of C-mitosis cells, multipolar anaphases, sticky and diffuse chromosomes together with the decrease in the mitotic index are defined as cytotoxic, and other nuclear abnormalities as genotoxic (Kanev et al. 2017). Chromosome abnormalities occur as a result of damage at the DNA level and are considered to be highly reliable analyzes for the evaluation of genotoxicity.

The abnormal cells in mitosis were observed at different levels in all treatment doses of extract and in the pesticides treatments. The highest number of aberrant cells was observed in Elandor® treatment with 88.1 aberrant cells, followed by the 50 ppm *E. rigida* extract with 84.1 and Goldplan® with 82.5 aberrant cells (Table 3). Total abnormal cell formation was the highest in applications where MI was high. Total abnormal cell counts were also observed to be the highest in these treatments as there was no toxic effect at the lowest dose of extract (*E. rigida* 50 ppm) and chemical pesticides. When the

Table 3. Type and percentage of mitotic abnormalities observed in the treatments with pesticides and different doses of *E. rigida* extract.

Doses (ppm)	Stickiness ± SD.	Fragment ± SD	Irregular Metaphase ± SD	C-Mitos ± SD	Laggard Chromosome ± SD	Irregular Anaphase ± SD	Pole Deviation ± SD	Bridge ± SD	Binucleus ± SD	Micronucleus ± SD	Other Anomalies ± SD	Total Aberrant Cell Number ± SD
Control (0)	23.4 ± 6.94 bcd	0.2 ± 0.63 a	12.5 ± 5.08 bc	1.4 ± 1.34 a	0.8 ± 0.91 ab	12.3 ± 5.27 b	6 ± 0.81 d	0.6 ± 0.84 ab	1.3 ± 1.05 ab	0.4 ± 0.69 a	0 ± 0 a	60.7
E. rigida 50	31.2 ± 13.72 d	0.7 ± 0.82 a	19 ± 11.46 cd	10.6 ± 11.85 b	2.3 ± 2.35 c	12.7 ± 4.85 b	2.9 ± 1.96 b	0.3 ± 0.48 ab	3 ± 1.76 bc	1.3 ± 0.82 b	0.1 ± 0.31 a	84.1
E. rigida 100	18.4 ± 7.80 bc	0.4 ± 0.69 a	9.5 ± 4.06 ab	3.6 ± 4.37 a	2 ± 2.10 bc	6.8 ± 4.61 ab	2.7 ± 2.66 b	0.2 ± 0.42 a	2.7 ± 2 abc	2.2 ± 1.54 c	0 ± 0 a	48.5
E. rigida 200	5.4 ± 2.22 a	0.4 ± 0.51 a	4.6 ± 1.77 a	0.6 ± 0.69 a	0.8 ±0.76 ab	3.2 ± 1.03 a	0.8 ± 0.78 a	0.3 ± 0.48 ab	1.2 ± 0.78 a	0.3 ± 0.48 a	0 ± 0 a	17.6
E. rigida 400	16.2 ± 8.13 b	0.3 ± 0.48 a	9.5 ± 4.30 ab	1 ± 0.69 a	0.4 ± 0.51 a	10.4 ± 6.71 b	3.6 ± 2.11 bc	0.5 ± 0.52 ab	3.5 ± 2.54 c	1 ± 0.66 ab	0.1 ± 0.31 a	46.5
Goldplan 400	25.1 ± 9.76 cd	0.6 ± 0.84 a	22.3 ± 9.42 d	3.1 ± 2.55 a	1.2 ± 0.91 abc	21.5 ± 8.80 c	5 ± 1.82 cd	1.2 ± 1.93 b	1.9 ± 1.1 abc	0.4 ± 0.51 a	0.2 ± 0.42 a	82.5
Elandor 200	27.5 ± 7.36 d	1.6 ± 2.01 b	23.3 ± 9.40 d	1.8 ± 0.91 a	1 ± 0.66 abc	23 ± 7.93 c	5.2 ± 2.82 cd	1 ± 0.66 ab	3.2 ± 2.2 c	0.5 ± 0.70 a	0 ± 0 a	88.1

Variability around the mean was represented as ± SD (Standart Deviation). Data having the same letter in a column were not significantly differed by LSD's multipli comparison test (P<0.05).

dividing cells were suddenly exposed to treatments after 48 hours, these cells, which were not affected by the lowest dose of extract and chemical pesticides at a toxic level, completed their division with abnormalities. On the other hand, in the cells exposed to high doses (100, 200 and 400 ppm) of plant extract, toxic effects and mitodepressive effects were observed, MI decreased and fewer abnormal cells were recorded.

The highest anomalies were chromosome stickiness (Figure 1a), irregular metaphase (Figure 1b), irregular anaphase (Figure 1c), pole deviations in the anaphase (Figure 1d), C-mitosis and aneuploidy (hipoploidy) (Figure 1e) (Table 3). Other anomalies observed in this study were fragmentation in the metaphase (Figure 1f), lagging chromosome (Figure 1g), bridges in anaphase (Figure 1h), binucleate cells (Figure 1i), micronuclei (Figure 1j). In addition to these anomalies, granulation in the prophase nucleus (Figure 1k), irregular prophase (Figure 1m), split in the interphase nucleus (Figure 1n), increases in the number of nucleolus in the nucleus (Figure 1o), nucleus erosion and granulation in the interphase nucleus (Figure 1p), nucleus vacuolization in the prophase (Figure 1q), multipolar anaphase with polyploidy (Figure 1r), polyploidy with C-Mitosis and fragments (s) were observed ($p<0.05$). Micronucleus (MN) occurs as a result of clastogenic and aneugenic effects (Andrade-Vieira et al. 2012; Adhikari 2019; Rosculete et al. 2020). Micronucleus analysis has an important role in assessment of the genotoxic and cytotoxic impacts of chemicals or pesticides (Karaismailoğlu 2015; 2017b). Disturbed ana-telophase and chromosome laggards may result from deformation of the spindle structure or degraded microtubules and remaining acentric chromosome fragments (Türkoğlu 2007; Andrade-Vieira et al. 2012; Pirdal and Liman 2019; Rosculete et al. 2020). Laggard chromosomes are considered indicators of spindle poisoning (Rank 2003). The induction of spindle disturbances in the cell of *A. cepa* by extracts may lead to aneuploidy and lagging chromosome(s) or micronucleus formation at the next stage of cell division. The lagging chromosome(s) may be lost or form nuclear membrane around itself thereby forming micronucleus (Grant 1978). The lagging chromosome(s) usually arises from irregular separation of chromosomes at anaphase thereby making some chromosomes to reach the poles before the other (Grant 1978; Pandey et al. 2014; Adhikari 2019; Rosculete et al. 2020). C-mitosis, binucleate cells, and increases in the number of nucleolus in the nucleus were also observed.

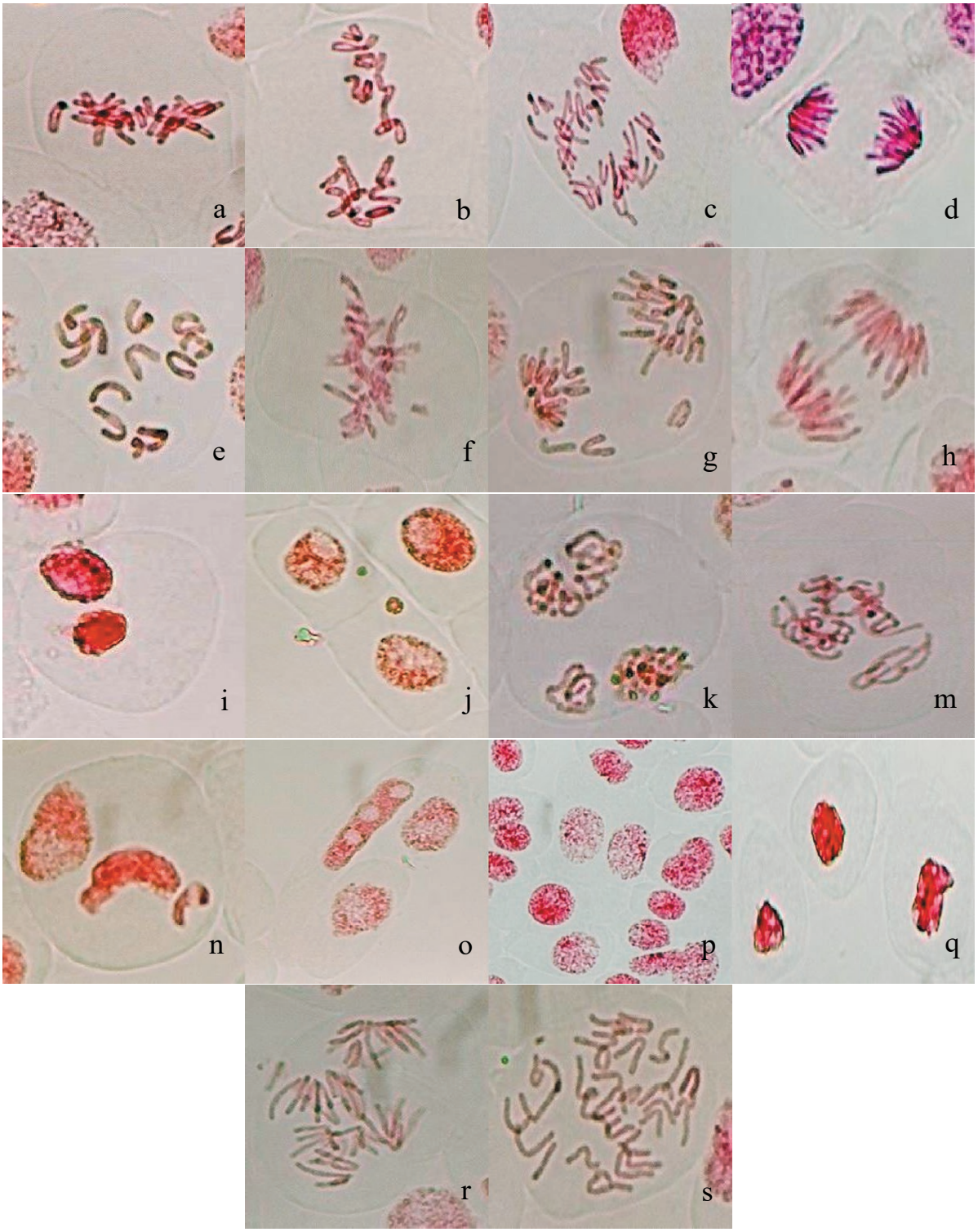

Figure 1. (a) Stickiness in Metaphase (100 ppm *E. rigida*) (b) Irreguler Metaphase (100 ppm) (c) Irreguler Anaphase, (100 ppm) (d) Pole deviation (Goldplan) (e) C-Mitosis and Aneploidy (100 ppm) (f) Fragments (400 ppm) (g) Laggard chromosome (50 ppm) (h) Bridge in Anaphase, (Goldplan) (i) Binucleus (200 ppm) (j) Micronucleus (50 ppm) (k) Granulation of nucleus (50 ppm) (m) Irreguler Prophase (100 ppm) (n) Nucleus deformation (Goldplan) (o) Increase in the number of Nucleolus in the Nucleus (Goldplan) (p) Nucleus erosion in interphase (200 ppm) (q) Vacualation of nucleus in interphase (200 ppm) (r) Multipolar Anaphase (Elandor) (s) Polyploidy with C- Mitosis and fragments (50 ppm) (10X40).

It is thought that the formation of binucleate cells may result from incomplete cell division or wall fusion as a result of halting protein synthesis (Sivas and Gökbayrak 2011). C-mitosis constitutes with stopping spindle form (Badr 1983). Mitotic toxicity affects spindle mechanism therewith C-mitosis is observed (Yüzbaşıoğlu 2003; Grisolia et al. 2004; Türkoğlu 2007; Sivas and Gökbayrak 2011; Andrade-Vieira et al. 2012; Pandey et al. 2014). The cytotoxic effects of urea, fungicides (Afugan, Carbendazim)(Yüzbaşıoğlu 2003; Bonciu et al. 2018), synthetic plant growth regulators (2,4-Dichlorophenoxyacetic acid)(Özkul et al. 2016), pesticides, herbicides, insecticides (Pentachlorophenol, Maleic Hydrazide, Dichlorvos and Glyphos)(Grant 1978; Kaymak 2005; Fındıklı and Türkoğlu 2010), heavy metal (Chromium=K2Cr2O7, Pb(NO3)2)(Güler et al. 2018; Girasun et al. 2019) and some plant extracts containing bioactive compounds (triterpenoids, tannins etc.)(Başaran et al. 1996; Shi et al. 2008) were observed from the occurrence of fragments on DNA doubled spindles (Çavuşoğlu 2019). Fragmentation might have arisen due to stickiness of chromosomes and consequent failure of separation of chromatids to poles. In addition to this, DNA double strand breaks induced by reactive oxygen species can lead to chromosome fragments (Adhikari 2019; Liman et al. 2020). It is claimed that the stickiness, bridges and fragments which are scored as indicators of clastogenicity in chromosomes are induced by chemicals regarded as clastogenic agents ($p<0.05$) (Rank 2003; Kaymak 2005; Sivas and Gökbayrak 2011; Andrade-Vieira et al. 2012; Adhikari 2019). These alterations can form an irreversible and genotoxic influence (Fiskesjö and Levan 1993). Stickiness aberration forms as a result of chromatid irregularity (Badr 1983). Stickiness in the chromosomes is an indication that chemical substance has a high toxicity, and may cause the death of cells by inducing unrecoverable damages (Fiskesjö 1985; Türkoğlu 2007; Andrade-Vieira et al. 2012; Pandey et al. 2014). Chromosome bridges may be due to the chromosomal stickiness and subsequent failure of free anaphase separation or may be attributed to unequal translocation or inversion of chromosome segments (Gömürgen 2005; Türkoğlu 2007; Sivas and Gökbayrak 2011). In this study, the highest stickiness in the chromosomes was seen in *E. rigida* 50 ppm, Elandor and Goldplan ($p<0.05$). Nucleus deformation increases depending on the increase in extract concentration, which indicates that the cells are affected cytotoxically and genotoxically, DNA synthesis is pressured. Vacuolisations were observed in *E. rigida* 200 ppm extract treatment, then this indicated that the chemical pesticides are more destructive and comprehensive mutagens, and those high concentrations of *E. rigida* 200 ppm extract give the same results. The anomalies occurred in all doses of *E. rigida* extract, but the highest anomalies was usually observed in *E. rigida* 50 and 100 ppm. Elandor and Goldplan are routinely used against control of *Hemiptera,* especially aphids, *Thysanoptera* and *Lepidoptera* (Öncüer 2004). When these pesticides were compared to extract of *E. rigida*, it was seen that even the high doses of *E. rigida* extract are more mutagenic ($p<0.05$). In this study, 200 and 400 ppm *E. rigida* extracts were found to be more cytotoxic and genotoxic than 200 ppm Goldplan or 400 ppm Elandor ($p<0.05$).

It is obvious from the results of the present study and based on the literature that the production of ATP is suppressed in the cells of *A. cepa* meristem tissue exposed to increased doses of *E. rigida* extract and chemical pesticides for 24-hour. Also, the metabolic activities in the cell slow down or stop. At the same time, depending on the clastogenic and aneugenic effects of plant extract and chemical pesticides, cells that tend to enter mitosis are eliminated. Thus, mitosis was suppressed in tissues and cells exposed to high doses and less abnormal cell formation were observed.

E. paralias, E. antiquorum and *E. chamaesyce* showed moderate antiviral activity (Shi et al. 2008). *E. paralias, E. maschallian* and *E. myrsinites* species have antiviral compounds. *E. pekinensis, E. peplus*, *Phyllanthus nanus* and *P. amarus* are effective against virus infections and *E.hirta* shows direct antiviral effects on HIV-1, HIV-2 and SIV (mac251) reverse transcriptase (RT) activity (Gyurıs et al. 2009; Alam et al. 2016). *E. rigida* plant did not cause gene change in bacteria such as TA 98 and TA 100, they showed mutagenic effects in human lymphocytes (Başaran et al. 1996). *E. rigida* extract may also have strong effects on viruses' capsid, reverse transcriptase (RT) and DNA/RNA structures. Although *E. rigida* extract is a natural and organic product, it is clear that it is more toxic, cytotoxic and genotoxic.

Therefore, the possibility of using *E. rigida* extract as an antiseptic for sterilization or disinfection of large and inanimate surfaces can be explored (Avcı et al. 2013).

According to the data obtained from cytotoxic and genotoxic tests in this study, the aqueous extract up to 50 ppm of *E. rigida* is seen to be promising for using as biopesticide purposes as an alternative to the chemicals we use in our experiments. However, studies on the evaluation of systematic toxicity and safety of *Euphorbia* species are very few. In the studies that have been conducted, only the organs that are targeted and side effects are emphasized (Huang et al. 2012). Although *E. rigida* extract is a natural and organic product, it is clear that it can be dangerous because it is more toxic, cytotoxic and

genotoxic than Goldplan and Elandor used as a chemical pesticide in agricultural control ($p<0.05$). In order to reach more information and certain conclusions on this subject, however, further research should be performed with different test systems.

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A preliminary report on X-chromosome sequence variability within Iranian population. Reporting new potential SNPs/ sequence variants as a source of population genetic markers

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Abstract. X-chromosome and its independent loci, recently has gained importance in genetic investigations that are concerned with diseases control-case studies, as well as in population genetic analyses and phylogenetic investigations. The present study was performed as a preliminary investigation on X-chromosome sequence variability as a source of genetic diversity within Iranian population and to investigate potential use of these sequences for differentiating the ethnic groups within the country. A limited random sample could be obtained from four different ethnic populations, which were sequenced for TEX-11 gene of the X-chromosome. At the beginning we used seven specific primers related to seven known SNPs within TEX-11 gene. Later on we searched for the other known as well as new potential SNPs or sequence variants in the studied samples. We could identify 87 potential SNPs or sequence variants. Genetic diversity analysis revealed the presence of five haplotype groups within the studied sequences. AMOVA revealed no significant genetic differentiation among sample driven from the ethnic populations. Moreover, Mantel test did not show significant association between geographical distance and genetic distance of these samples, indicating that gene flow occurred among these populations with no geographical obstacle. This is the first report on X-chromosome sequence diversity analysis in Iran.

Keyword: X-chromosome, SNPs, population, haplotype.

INTRODUCTION

Population genetic study produces data on genetic structure and genetic diversity within target populations and investigates genetic admixture and gene flow versus reproductive isolation among the studied populations. It

also may identify specific genotypes or genes in local geographical or ethnic populations (Freeland et al. 2011).

X-chromosome markers are of potential use in population and forensic genetic studies (Zhang et al. 2015), due to their peculiar transmission property (Garrigan et al. 2007). These genetic markers are transmitted between both sexes in each generation, and they may portrait a different picture from uniparental genomes. These molecular markers are sensitive to the evolutionary processes like population substructure or fragmentation and genetic drift, as their effective population size is reduced in relation to autosomes (Garrigan et al. 2007; Zhang et al. 2015).

Nowadays, the number of genetic investigators interested in applying X-chromosome markers in genetic studies grows as these markers show higher efficiency parameters than autosomes in special kinship investigations involving mainly female offspring. These genetic X chromosome markers are X-STRs, X-SNPs and more recently X-Indels, that also may be utilized in studying the genetic structure of human populations, and investigating the ancestry proportions in the admixed populations as well as for forensic investigations (Garrigan et al. 2007; Zhang et al. 2015).

The present investigation was carried out as a side work during investigation on TEX-11 gene and its association with infertility. Therefore, we also used TEX-11 sequences as a population genetic marker to produce preliminary data on sequence variability and gene flow in a small sample size of Iranians who were randomly chosen from four ethnic populations. This is the first report on X-chromosome sequence diversity.

MATERIAL AND METHODS

Samples studied

In total 30 persons, were randomly selected for sequencing. The studied samples were taken from four ethnic Iranian populations, 1- Turk, 2- Fars, 3- Lor, and 4- Kurd.

Gene and SNPs selection

TEX11 is one the most important genes in the spermatogenesis process that has tissue specific expression. It is expressed in pancreas and testis only. The SNP selection was based on PHYRE2 online software and by determining the rate of amino acids conservation and protein structure sensitivity to amino acid alteration (Yang et al. 2015). The SNPs chosen were not studied before.

DNA extraction and PCR details

The blood samples were obtained from Jihad center of Qom University (informed consent was obtained from all participate). The standard salting out method was used for extraction of genomic DNA from blood samples. The quality of DNA samples were examined by 1% agarose gel electrophorese. Target TEX11 gene was amplified by PCR reaction, while forward as well as reverse primers were designed by oligo7 version 7.56 (Table 1).

PCR was done by thermo cycler system (Genetix Biotech, Australia), having the initial denaturation of

Table 1. Seven different TEX-11 gene regions for sequencing.

Primers information			
Forward	rs775667438	5’ GCATGGCATCTATCTCTCTG 3’	TM= 57
Reverse		5’ GGTGAATTATGGGTGTTCTC 3’	TM= 57
Forward	rs746940663	5’ TGCCCACGATACCTACTG 3’	TM= 54
Reverse		5’ GACTGAATATGGACAGAGGA 3’	TM= 54
Forward	rs6525433	5’ AAATGCTAACTGTTGCTTTT 3’	TM= 55
Reverse		5’ CCCACGATACCTACTGACTC 3’	TM= 53
Forward	rs756553436	5’ CAACATCAAGGTGCTCGCAG 3’	TM= 57
Reverse		5’ GAAGATGCCTGTCACTGTGG 3’	TM= 58
Forward	rs867296518	5’ATCAGCGATGACATTTCCCTAC 3’	TM= 57
Reverse		5’ GAGAGGGAGACAATAGACCGAG 3’	TM= 57
Forward	rs977597709	5’ CCCAATTTGTGGGATGTGGACAG 3’	TM= 60
Reverse		5’ TCTGTTGGGTTTCATTTCCTGACG 3’	TM= 59
Forward	rs1056191384	5’ CTTGTTCAAAGGTACACAGC 3’	TM= 54
Reverse		5’ CATGTAACTTCACTGGATCTCG 3’	TM= 54

95°C for 4 min. This was then followed by 40 cycles of denaturation at 95°C for 30 s, and annealing at different temperatures according to different primers used (Table1) for 30 s, and extension at 72°C for 45 s. The final extension was done at 72°C for 10 min.

PCR reaction was carried out in total volume 25 µl containing 3 µl of DNA samples, 22 µl Master Mix (17.2 µl H2O, 2 µl Buffer 10X, 0.3 µl MgCl2 100 mM, 0.4 µl dNTP-Mix 40 mM, 0.8 µl Forward primer 14.72 ng/µl and 0.8 µl Reverse primer 12.77 ng/µl) and 0.5 µl Taqpolymerase (5 u/µl).The PCR fragments were separated by 1% agarose gel electrophoresis and visualized with green viewer staining (5u/µl).

Data analyses

Sequence alignment and curation was done by MUSCLE program implemented in MEGA 7 software (Tamura et al. 2012). Multidimentional scaling methods (MDS) was performed on these sequences to investigate the ethnic populations genetic differentiation. This can be taken as indicating gene flow and genetic admixture among ethnic groups. For this, Kimura 2-parameters were used as genetic distance of the studied samples.

Haplotype groups and haplotype diversity were determined by TCS networking as performed in POPART (Population Analysis with Reticulate Trees) program (http://popart.otago.ac.nz). Significant genetic difference among the ethnic populations was studied by analysis of molecular variance (AMOVA) and by calculation PHIST as implemented in POPART ver. 3 (Librado and Rozas 2009). The Mantel test was used to investigate association between genetic distance and geographical distance of the studied ethnic groups (Podani 2000).

RESULTS

Sequence variability and haplotype groups

In total we obtained 1935 bp length DNA after alignment and curation. The nucleotide diversity obtained was pi = 0.014, the number of segregating sites = 206, the No. of parsimony-informative sites was 6, and Tajima's D statistic was D = -1.92938 p (D >= -1.92938) = 0.984141. All these results indicated a low degree of nucleotide substitution and that these substitutions are not under selective pressure.

The studied samples differed in sequences as they formed different clusters/ clades in TCS network (Fig. 1). Some of the sequences were placed close to the outgroup (main sequence = No. 1 in Fig. 1), and formed the haplotype group A, due to sequence similarity. However, the samples within this haplotype group, also revealed some degree of sequence variability.

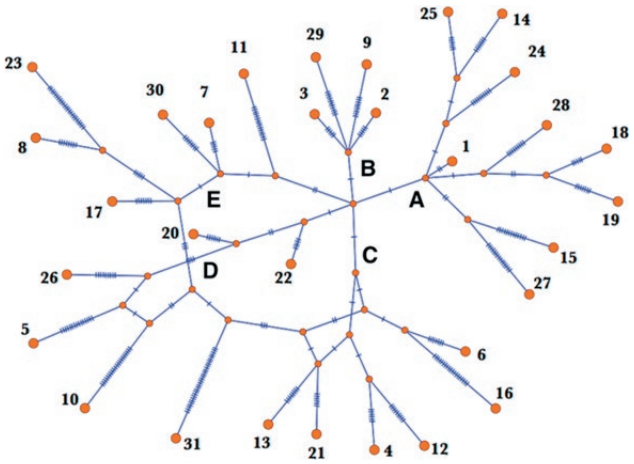

Figure 1. TCS network of the studied sequences showing five major haplotype groups.

The samples within B and C haplotype groups were placed close to the A group. Members of these haplotype groups also differed in the number of nucleotide substitutions. However, haplotype groups D and E were placed far from the A group, and therefore, are much more deviant from the reference sequence.

The highest number of nucleotide substitutions occurred in the sequence 31 in haplotype group D (30 substitutions), followed by sequence 23 of the haplotype group E (23 substitutions).

Gene flow and population admixture

We investigated the population genetic parameters based on ethnic populations from which our samples were collected (Table 2 and 3).

AMOVA performed did not produced significant difference among the studied ethnic populations (Phist = 0.2, P = 0.26). AMOVA revealed that only 3% of total genetic variation occurred among the studied ethnic populations, while 97% was due to with population genetic variability. Therefore, it indicates that these ethnic populations are not genetically differentiated and that they have/had frequent gene flow. This is also supported by MDS plot based on ethnic groups (Fig. 2) which also reveals high degree of genetic admixture among the studied ethnic populations.

The Mantel test did not produce significant association between genetic distance and geographical distance of ethnic populations studied (r = 0.10, P = 0.3).

Table 2. Genetic diversity parameters determined in the studied SNPs.

	No. Haplotypes	Haplotype diversity	Nucleotide diversity	Polymorphic Sites
SNP1	8	0.71	0.04	287
SNP2	7	0.40	0.03	68
SNP3	5	0.30	0.03	37
SNP4	2	0.01	0.005	3
SNP5	6	0.50	0.03	40
SNP6	8	0.70	0.04	250
SNP7	2	0.02	0.006	10

Table 3. Details of ethnic populations in which SNP sequences were potentially associated with the male infertility

Nomber	SNP	Fars	Lor	Kurd	Turk
SNP1	Rs6525433	-	+	-	-
SNP2	Rs1056191384	-	+	+	-
SNP3	Rs746940663	+	-	-	-
SNP4	Rs977597709	+	+	-	-
SNP5	Rs775667438	+	-	+	+
SNP6	Rs867296518	+	-	-	+
SNP7	Rs756553436	-	-	-	-

Therefore, geographical distance was not an obstacle for migration and gene flow for the studied populations.

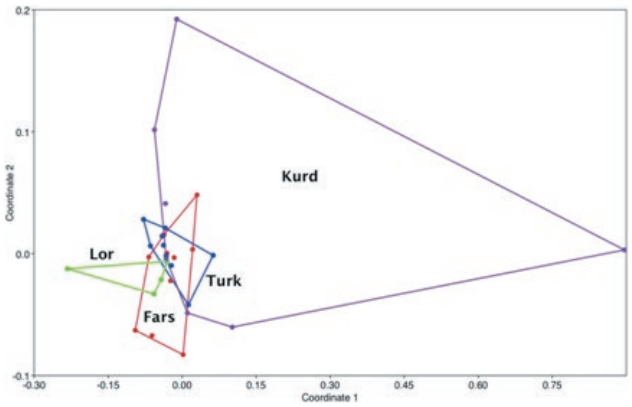

Figure 2. MDS plot of the studied sequences based on ethnic group samples showing admixture of the samples.

New potential SNPs / sequence variants within TEX-11 gene

In total we found 87 SNPs and sequence variants within TEX-11, details of which are provided in Table 4. Out of 87 SNPs observed, 18 SNPs were previously reported as RS in NCBI (Table 4), while the rest of them are new reports. However, at present we consider these SNPs as polymorphism within Iranian samples studied.

Details of the SNP nucleotides are also provided for each sequence position. In some cases multiple substitutions were observed within the studied samples (Fig. 3).

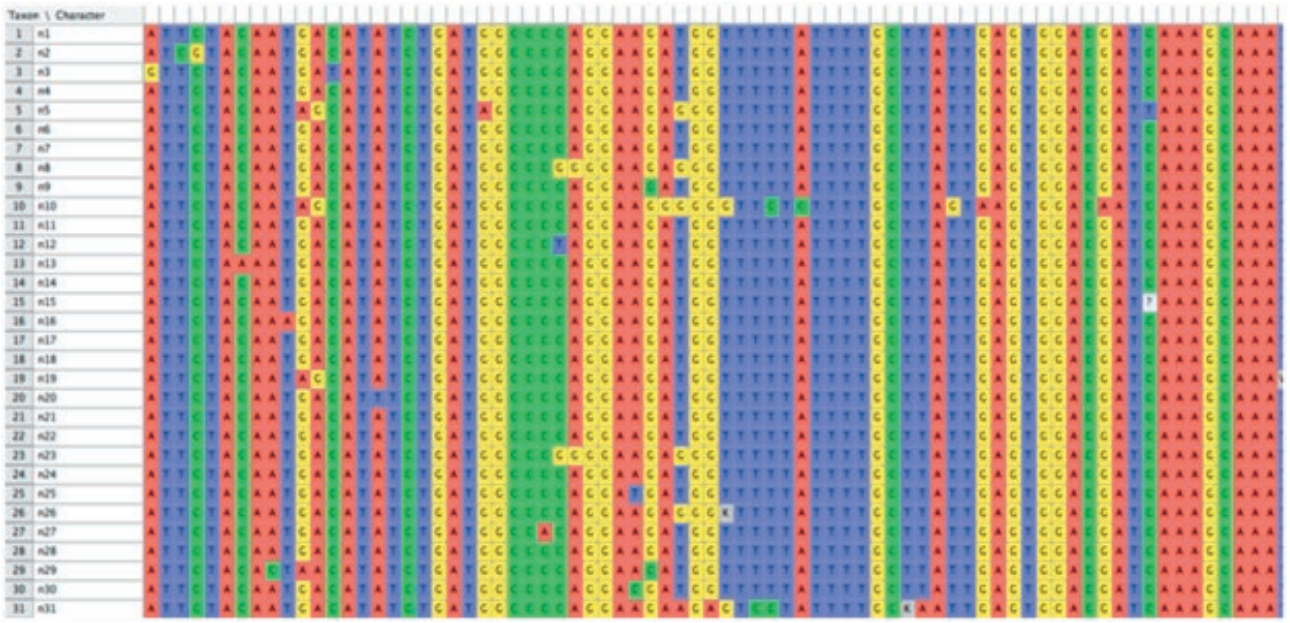

Figure 3. SNP diversity within TEXT-11 gene in the studied samples.

Table4. Details of SNPs/sequence polymorphism with TEXT-11 gene in the studied samples and NCBI.

Reference SNP	Alleles	Position in sequence	NO	Reference SNP	Alleles	Position in sequence	NO
-	T/G	1615	81	-	T/A	49	1
-	T/C	1617	82		A/T	52	2
-	C/T	1620	83		A/G	59	3
-	G/T	1622	84	RS181582479	G/A	95	4
-	T/C	1624	85	-	G/T	442	5
-	C/T	1627	86	-	A/C/G	485	6
-	G/A	1631	87	--	G/T/A	488	7
-	A/G	1632	88	-	A/T/A	490	8
-	G/A	1635	89	RS375000923	C/T(A)	491	9
--	T/G	1636	90	-	T/A/C	492	10
-	T/C	1638	91	-	C/T/A	493	11
-	T/C	1640	92	-	T/G	494	12
-	A/C	1642	93	RS755626796	G/A(T)	495	13
-	T/A	1651	94	-	A/C/G	496	14
-	G/A	1653	95	-	A/C/T	497	15
-	T/G	1654	96	-	T/G/C	502	16
-	T/G	1655	97	RS1296061122	C/T(A)	505	17
-	T/C	1661	98	RS1247146060	T/C(A)	506	18
-	A/C	1665	99	-	C/A/T	507	19
RS1186389277	C/T	1666	100	-	T/C	508	20
-	G/T	1678	101	RS1365016323	C/T(A)	509	21
-	G/A	1682	102	-	C/G/A	512	22
-	A/G	1691	103	RS748927377	A/G	513	23
-	G/A	1695	104	-	G/C	514	24
-	G/T	1697	105	RS1341488009	T/C(G)	515	25
-	T/G	1699	106	-	A/C/T	516	26
-	G/C	1700	107	-	T/C	521	27
-	A/C	1703	108	-	C/T	525	28
-	T/G	1708	109	-	A/C	534	29
-	A/C	1710	110	-	A/C	620	30
-	A/C	1711	111	-	T/A	646	31
-	A/G	1712	112	-	A/G	700	32
-	G/C	1713	113	-	A/T	721	33
-	A/C	1714	114	-	T/C	752	34
-	A/T	1717	115	-	A/G	766	35
-	G/C/A	1721	116	RS181582479	C/T	811	36
-	G/C	1729	117	-	C/A/T	867	37
RS993988398	A/G	1730	118	-	G/T	892	38
-	A/G	1731	119	-	C/G	906	39
-	A/G	1739	120	-	T/C	908	40
RS113894957	A/G	1744	121	-	G/A	953	41
-	A/C	1750	122	-	T/G	957	42
-	A/T	1754	123	-	A/C	974	43
-	T/A	1760	124	-	T/A	982	44
-	G/C	1764	125	-	T/C	1032	45
-	T/A	1768	126	-	G/A	1041	46
-	T/A	1774	127	Rs774596712	A/G	1068	47
-	C/T	1780	128	-	T/C	1070	48
-	T/C	1781	129	-	C/G	1071	49

Reference SNP	Alleles	Position in sequence	NO	Reference SNP	Alleles	Position in sequence	NO
-	G/A	1782	130	-	C/A	1074	50
-	T/C	1788	131	-	A/C	1144	51
-	A/C/T	1789	132	-	C/T	1412	52
-	T/A	1826	133	-	T/G	1413	53
-	G/C	1827	134	-	G/T	1414	54
-	C/T	2332	135	-	A/G	1429	55
-	T/A	2335	136	RS1188518295	G/A	1430	56
-	T/C	2340	137	-	A/G	1431	57
-	A/T	2343	138	-	C/T	1441	58
-	T/A	2353	139	-	A/T	1444	59
-	G/A	2357	140	-	G/A	1451	60
-	G/A	2359	141	-	A/G	1456	61
-	G/A	2360	142	-	T/G	1460	62
-	T/C	2364	143	-	C/T	1461	63
--	T/A	2374	144	-	C/A	1486	64
-	G/C	2399	145	RS1161677215	C/T(G)	1488	65
-	C/T	2412	146	-	A/G	1489	66
-	A/T	2414	147	-	A/T/C	1495	67
-	T/A	2417	148	-	G/C	1496	68
-	G/C	2421	149	-	G/C/T	1497	69
-	A/T	2430	150	-	A/T	1499	70
-	T/A	2435	151	-	C/T	1501	71
-	A/T	2436	152	-	T/C	1599	72
-	C/T	2445	153	-	T/A/C	1600	73
-	C/A	2454	154	-	T/G/A	1601	74
RS1256779249	A/T	2455	155	-	A/G	1603	75
RS993488245	G/C	2474	156	-	C/T	1605	76
-	T/A	2551	157	-	C/A/T	1607	77
-	C/G	2556	158	-	A/T	1608	78
-	C/T	2644	159	-	A/G/T	1612	79
-	G/T	2645	160	RS1452980045	G/T(A)	1614	80

DISCUSSION

We observed almost a moderate level of sequence variability in X-chromosome. In a similar investigation, Nachman et al. (1998), sequenced 11,365 bp from introns of seven X-linked genes in 10 humans, one chimpanzee, and one orangutan and reported the average value for pi as low as 0.063% with Standard error = 0.036%. These authors reported a positive correlation between heterozygosity and rate of recombination. In total it was suggested that the joint effects of selection and linkage are important in shaping patterns of nucleotide variation in humans (Nachman et al. 1998).

The present study revealed some degree of genetic admixture and gene flow among the ethnic populations studied. In a similar study Zhang et al. (2015), used 34 X-Chromosome markers (18 X-STRs and 16 X-Indels), to investigate genetic variability and admixture in ethnic populations of China. They reported genetic variability of 0.4-0.7 for the studied populations, while Phylogenetic tree and PCA analyses revealed a clear pattern of population differentiation. The study suggested that geographic isolation and interactions play significant roles in differentiation of genetic constitution of ethnic groups.

The use of the X chromosome in population genetics is still in its infancy. It has already proved its worth in studies of the early history of modern Homo sapiens (Harris and Hey 1999), but in most research areas its potential remains largely untapped. Haplotypes obtained based on coding as well as non-coding sequences in X-chromosome are of genetic analysis importance, particularly in genetic variability analysis (Kaessmann et al. 1999), as also revealed in present study

In conclusion, the present study revealed potential use of coding as well as non-coding sequences in genetic variability studies.

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Morphological and genetic variation of *Chamaerops humilis* (Arecaceae) in relation to the altitude

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Abstract. The Mediterranean dwarf palm (*Chamaerops humilis* L.) is native to Western and Central Mediterranean. Since classical times this species has been cultivated and several varieties have been described on material of unknown origins. In this study, plants grown from seeds collected in the wild from seven populations spread along the Mediterranean basin were cultivated under the same environmental conditions, investigated morphologically and genetically by screening the polymorphism of ten SSR loci. Two groups are clearly separated, the populations growing at low altitudes and those living above a thousand meters of altitude. Due to morphological, geographic and environmental isolation, here it is proposed to discriminate the populations growing at high altitude on the Moroccan High Atlas and Anti-Atlas as a distinct subspecies.

Keywords: European fan palm, dwarf fan palm, morphology, diversity, SSR markers, Morocco.

INTRODUCTION

The Mediterranean dwarf palm, *Chamaerops humilis* L. (Arecaceae) is one of the best known and typical species of the Mediterranean basin. This is due to its wide distribution and to its numerous uses since the dawn of civilization. *C. humilis* has been studied in relation to morphological and genetic diversity (Giovino *et al.* 2014, Giovino et al. 2015a, Guzmán *et al.* 2017), seed lipids composition (Giovino *et al.* 2015b), ethnobotany (Okkacha *et al.* 2013).

This species naturally occurs in Western and Central Mediterranean. It is widespread in the Iberian Peninsula, Morocco, Algeria, South France, Sardinia, Tunisia, Sicily and Peninsular Italy (Euro+Med 2006, Castroviejo and Galan 2007, Pignatti 2017). The eastern distribution limit of this species lies in Calabria, Italy. Linnaeus (1753) reported *C. humilis* as common in Spain.

The original material used for lectotypification of this name is presumptively collected in Europe (Moore and Dransfield 1979).

To date, the dwarf palm is considered a species threatened by human activities and environmental and climatic changes and for this reason deserves a special attention in conservation management strategies (Blach-Overgaard *et al.* 2010; Giovino *et al.* 2016).

Chamaerops humilis is extremely variable in height, leaf colour, presence of thorns, size and shape of fruits (Beccari 1921, Zagolin 1921, Maire 1957). This large morphological variation is at the basis of the description of numerous varieties. Despite the Kew Checklist of Palms (Govaerts and Dransfield 2005) reports more than 20 intra-specific epithets among varieties and sub-varieties, Giovino *et al.* (2014) reports that many morphological traits appear to be related to environmental conditions. This scenario is complicated by the fact that a large part of known varieties has been described on cultivated plants of unknown origin. Whilst a great number of names are used in the horticultural field, only two varieties are widely accepted in floras: *C. humilis* L. var. *humilis* and *C. humilis* var. *argentea* André (= *C. humilis* var. *cerifera* Becc.). They are mainly distinguished by leaves color: *C. humilis* L. var. *humilis,* has green leaves and *C. humilis* var. *cerifera* Becc. has grey and waxy leaves (Maire 1957).

The aim of this contribution is to investigate the taxonomic value of *C. humilis* var. *argentea* André and to clarify if the morphological variability observed in high altitude populations studied in High Atlas and Anti Atlas in Morocco is merely due to environmental factors or if it is of genotypic nature and transmitted to progenies.

MATERIALS AND METHODS

A preliminary assessment of the morphological variability of *C. humilis* in all the countries where it naturally occurs was performed. We were not able to find any wild individual in the Maltese archipelago, where this species was recorded by Haslam *et al.* (1977). After this preliminary evaluation, seven representative populations were chosen for this study.

In order to exclude variability due to the environment, plants grown from seeds collected in nature were studied *ex-situ* under the same environmental conditions in a collection field at the CREA-DC Research Center - Bagheria (N Sicily). These seeds were collected from three populations in Morocco (Terketen, BeniMellal, and Touama) (Figure 1), one population in Spain (Valencia), one in Algeria (Sidi Belattar near Mostaganem), one in Tunisia (Cap Serrat), and one in Sardinia, Italy (Porto Tangone) (Table 1). Seed collections were done on at least six individuals occurring in the central part of wild populations. In December 2013, seeds were treated with either sulphuric acid, water or mechanical scarification as described in Giovino *et al.* (2015b). After, seeds were germinated on humid sand for about 100 days (MGT) in a cold greenhouse with temperatures between 12 °C and 16 °C at relative humidity of 90%. After germination plants were transplanted on pots of 1.6 ℓ containing a mixture of sand (70%), red soil (25%) and commercial garden soil (5%). Pots were maintained on open air from April to November and irrigated with 1 dripper 2 ℓ/h providing 2 min irrigation per day for three days a week. After two years plant were transplanted in 7 ℓ pots containing the same substratum.

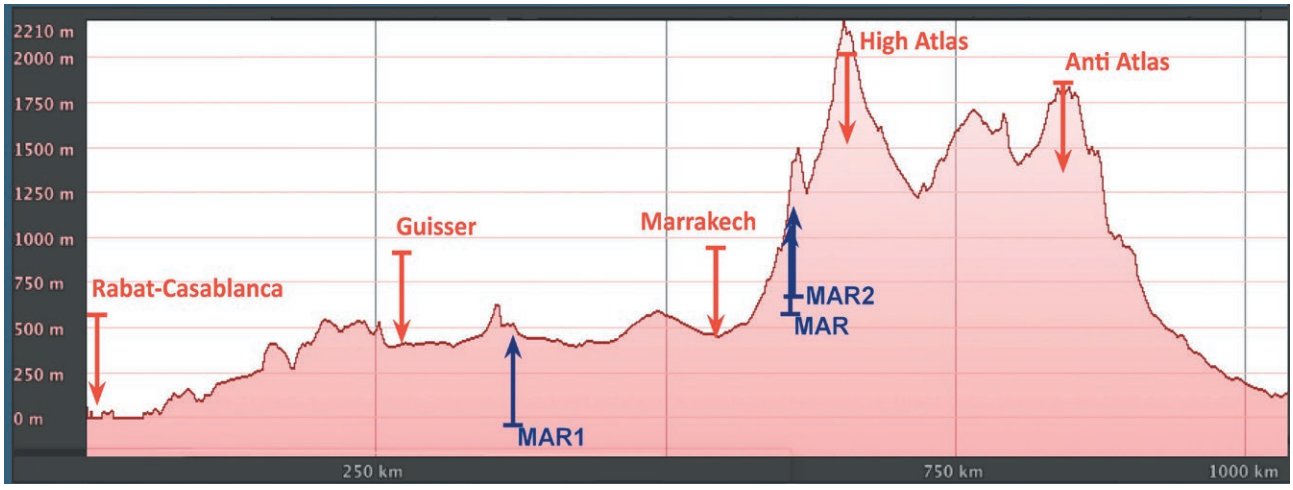

Figure 1. Elevation of the collecting localities in Morocco. MAR: Terketen; MAR1: Beni Mellal; MAR2: Touama.

Table 1. Sampled populations, environmental characteristics and seeds collection data.

Code	Locality	Latitude	Longitude	Altitude m a.s.l.	Habitat	Bioclimate	Date	Collector
MAR	Terketen, Morocco	31°27'44.47"N	7°24'23.39"W	1420	Mediterranean steppe	Humid	21.10.2012	A. Giovino
MAR1	Beni Mellal, Morocco	32°25'54.78"N	6°30'41.76"W	430	Mediterranean maquis	Semiarid	21.10.2012	A. Giovino
MAR2	Touama, Morocco	31°31'46.14"N	7°28'59.82"W	990	Mediterranean steppe	Humid	21.10.2012	A. Giovino
SPA	Valencia, Spain	40°16'15.12"N	0°17'12.04"E	130	Mediterranean maquis	Semiarid	15.10.2012	P. Ferrer Galego
ALG	Sidi Belattar, Algeria	36°01'43.07"N	0°09'03.03"E	200	Mediterranean maquis	Semiarid	5.9.2013	A. Mostari
TUN	Cap Serrat, Tunisia	37°11' 55.8"N	09°15'45.9"E	20	Mediterranean maquis	Humid	5.9.2013	R. El Mokni
SAR	Porto Tangone, Italy	40°28'19.23"N	8°22'52.98"E	50	Mediterranean maquis	Semiarid	25.06.2013	A. Giovino

For morphological analysis, the selection of characters was done on the basis of Rhouma (1994, 2005), Hammadi *et al.* (2009), Rizk and El Sharabasy (2007).

Measures were done in spring 2018 with a digital caliper. For each character three measures were performed and their arithmetic average registered. Per each population 31 individuals were measured. On the whole 12 quantitative characters were measured; nine continuous: Height of the stem (cm), Height of the plant (cm), Crown diameter (cm), Stem diameter (mm), Petiole length (cm), Petiole width (cm), Leaf length (cm), Leaf width (cm), Leaf thickness (mm) and three discrete ones: No. of leaf segments, No. thorns on the petiole, and wax coverage density. This latter was estimated on percentage of coverage on the upper surface of the leaf.

The measures used for statistics are presented in the Supplemental data (File 1). According to the methodology adopted in Giovino *et al.* (2015c), Domina *et al.* (2017a, 2017b), and Domina (2018) these characters were subjected to a Principal Component Analysis, with the individuals a priori assigned to the eight populations (Figure 2). Each character was also subjected to univariate analysis (analysis of variance or Kruskal–Wallis test with corrections for multiple comparisons, Pearson correlation coefficients, Tukey's HSD, honestly significant difference, test and Bonferroni, respectively),

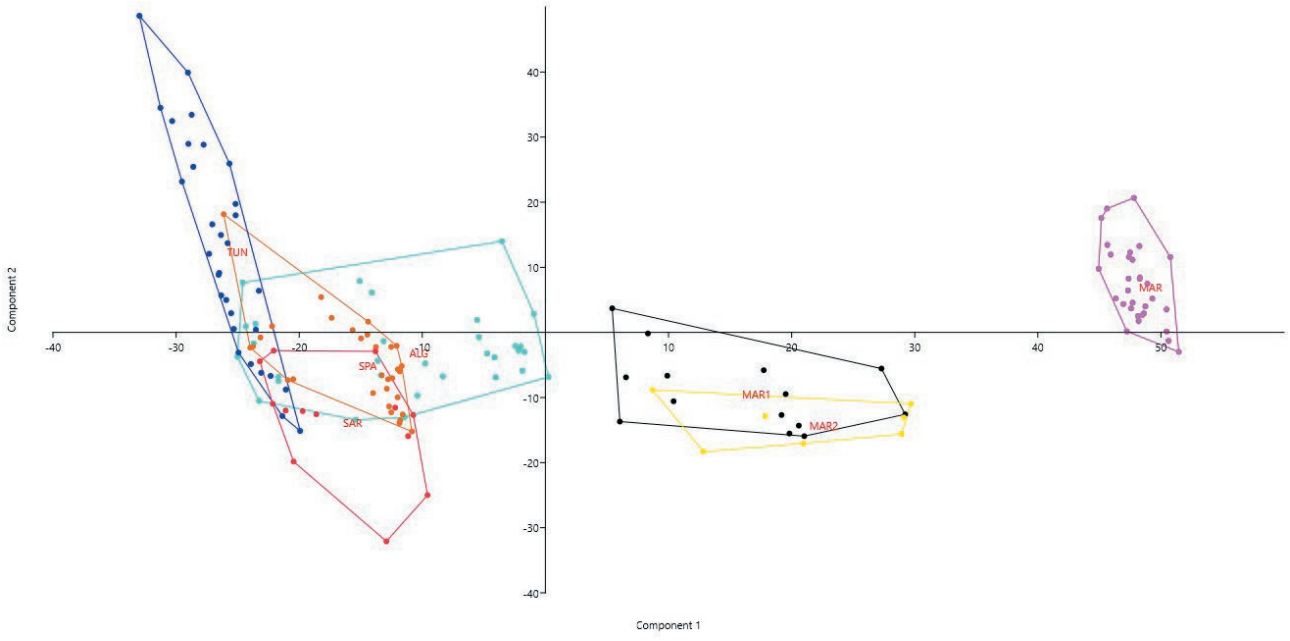

Figure 2. Principal components analysis based on the 12 considered morphological characters, with 7 a priori defined groups based on the geographical distribution of the sampled populations. PC1 Eigenvalue 645.724, % variance 76.48, PC2 Eigenvalue 132.387, % variance 15.68. MAR: Terketen, Morocco; MAR1: Beni Mellal, Morocco; MAR2: Touama, Morocco; SPA: Valencia, Spain; ALG: Sidi Belattar, Algeria; TUN: Cap Serrat, Tunisia; SAR: Porto Tangone, Sardinia, Italy.

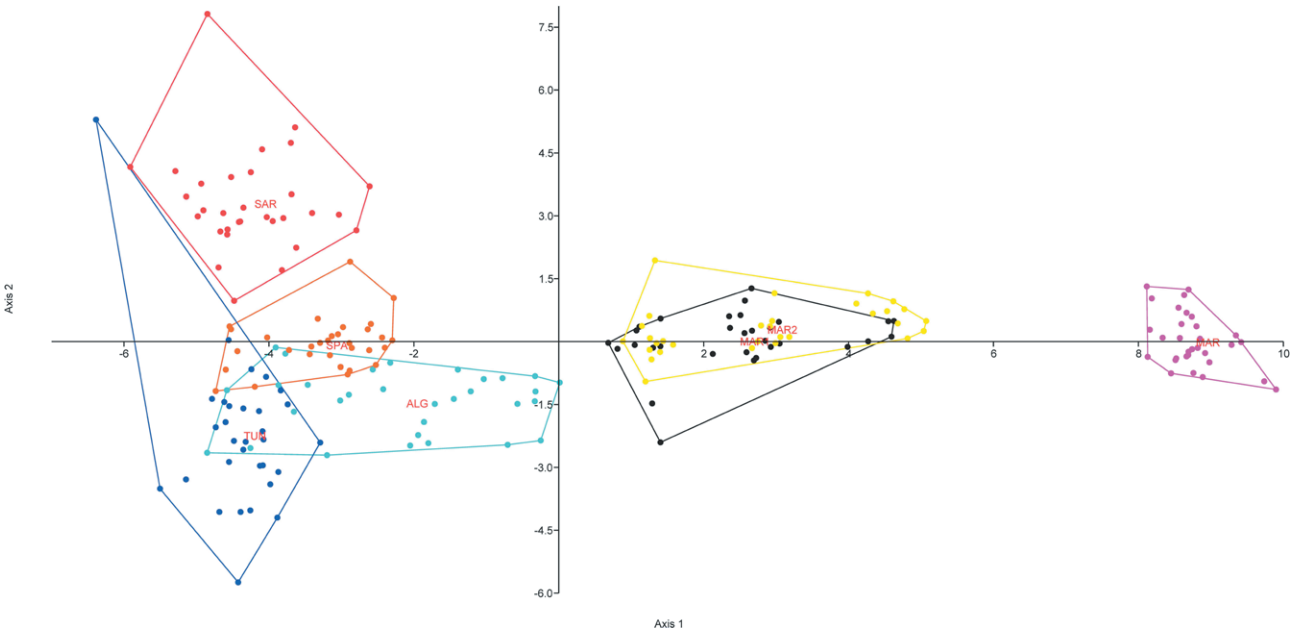

Figure 3. Discriminant analysis based on the 12 considered morphological characters, with 7 a priori defined groups based on the geographical distribution of the sampled populations. Axis 1: Eigenvalue 22.424, % variance 74.11; Axis 2: Eigenvalue 5.635, % variance 18.62. MAR: Terketen, Morocco; MAR1: Beni Mellal, Morocco; MAR2: Touama, Morocco; SPA: Valencia, Spain; ALG: Sidi Belattar, Algeria; TUN: Cap Serrat, Tunisia; SAR: Porto Tangone, Sardinia, Italy.

using PAST version 4.03 (Hammer et al. 2001; Hammer 2020). Pearson correlation coefficients (r) among the 12 characters measured are presented in the Supplemental data (File 2). A discriminant analysis for the seven a priori recognized groups was performed. The scatter plot of specimens along the first two canonical axes is shown (Figure 3). The range of each continuous numerical character is represented using box-and-whisker plots (Figure 4).

For molecular analysis a total of 35 genotypes were used for the characterization: 5 from Terketen (Morocco); 5 from Beni Mellal (Morocco); 4 from Touama (Morocco); 6 from Valencia (Spain); 6 from Mostaganem (Algeria); 3 from Cap Serrat (Tunisia), and 6 from Porto Tangone (Sardinia, Italy).

Genomic DNA was extracted from 40 mg of dried leaf sample using Doyle & Doyle (1987) protocol. A set of 10 microsatellite markers was employed, including 6 microsatellites showing trinucleotide repeats (Arranz *et al.* 2013) and 4 showing dinucleotide repeats recently isolated in fan palm by Giovino *et al.* (submitted 2020) following PCR conditions and thermal cycles reported in Giovino *et al.* (2014) and Giovino *et al.* (submitted 2020) (Table 2). PCR products were analyzed using an ABI 3130 Genetic Analyzer (Applied Biosystems) and allele sizes were established using GENEMAPPER, version 4.0 software (Applied Biosystems).

Basic genetic parameters including the number of alleles per locus (Na), observed (Ho) and expected heterozygosity (He), the total number of null alleles (Fnull), the polymorphic information content (PIC) value and the deviation from the Hardy-Weinberg equilibrium (HWE), inferred by sequential Bonferroni correction, in the 36 selected genotypes, were calculated using CERVUS 3.0 software (Marshall et al. 1998; Kalinowski et al. 2007).

A Neighbor-Net graph was constructed based on calculations of Reynold's genetic distances with SPLITSTREE (Huson and Bryant 2006) in order to study the genetic diversity and relationships between palm genotypes (Figure 5).

RESULTS

Morphological characterisation

Single morphological characters (Figure 4) show continuous variation and do not distinct population groupings. The population from Cap Serrat shows the largest intra-population variation. Only the leaf length distinguishes, in part, some populations from the others. Univariate analysis shows that this character discriminates Moroccan populations from the others.

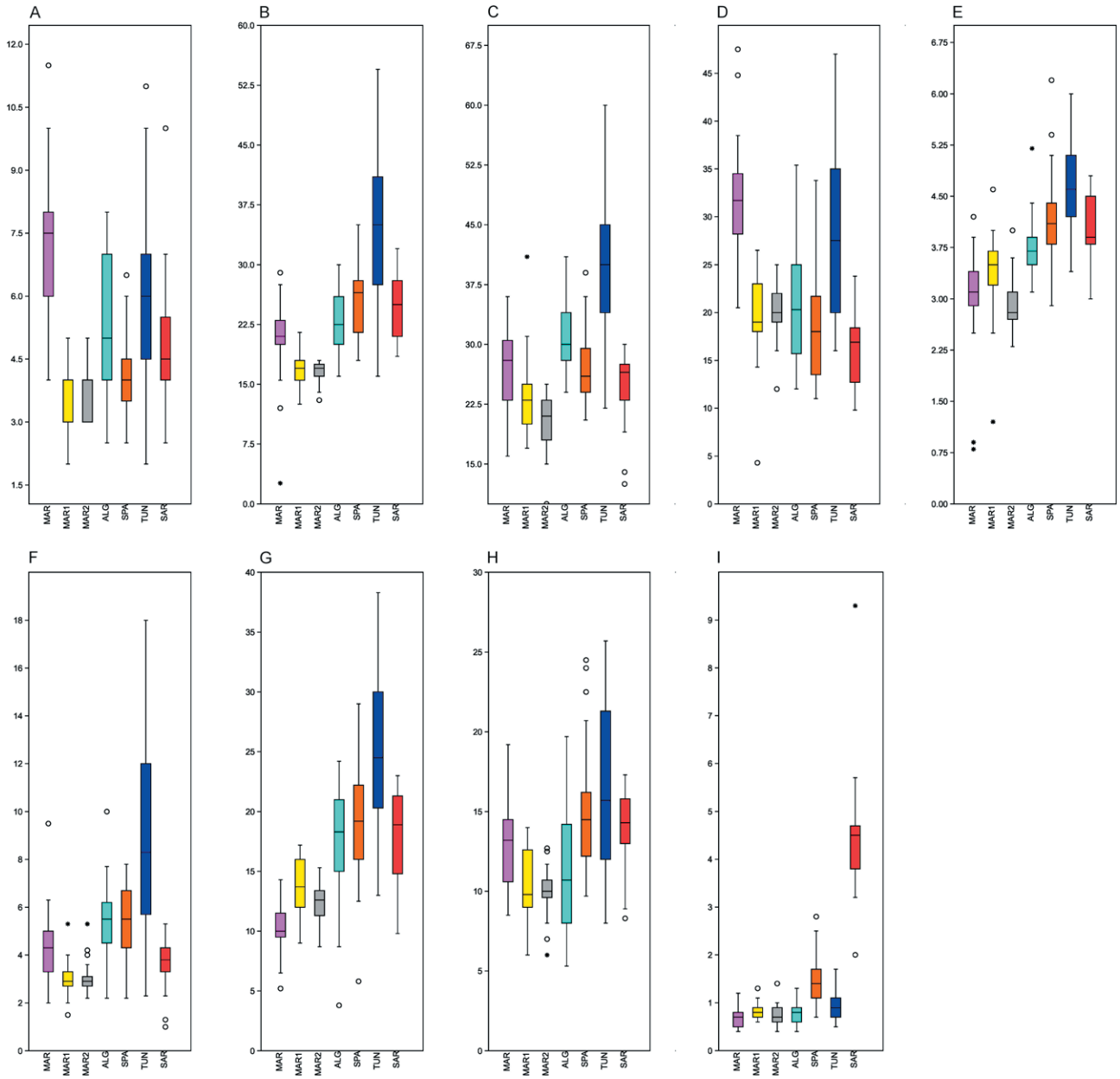

Figure 4. Box-plots of the 9 continuous morphological characters considered (A: height of the Stem (cm); B: Height of the Plant (cm); C: Crown diameter (cm); D: Stem diameter (mm); E: Petiole length (cm); F: Petiole width (cm); G: Leaf length (cm); H: Leaf width (cm); I: Leaf thickness (mm). For each sample, the 25–75% quartiles are drawn using a box. The median is shown with a horizontal line inside the box. The whiskers are drawn from the top of the box up to the largest data point less than 1.5 times the box height from the box, and similarly below the box. Values outside the inner fences are shown as circles, values further than 3 times the box height from the box are shown as stars. MAR (fuchsia): Terketen, Morocco; MAR1 (yellow): Beni Mellal, Morocco; MAR2 (grey): Touama, Morocco; ALG (light blue): Sidi Belattar, Algeria; SPA (orange): Valencia, Spain; TUN (blue): Cap Serrat, Tunisia; SAR (red): Porto Tangone, Sardinia, Italy.

The principal components analysis (Figure 2) and the discriminant analysis (Figure 3) show three well distinct groups across the populations studied. The cases correctly classified by discriminant analysis according to the groups assigned a priori were 91.4%.

Molecular characterisation

A total of 71 SSR alleles were identified across the 10 loci (Table 2) in 35 dwarf palm genotypes and all individuals were differentiated. Locus37 showed the highest

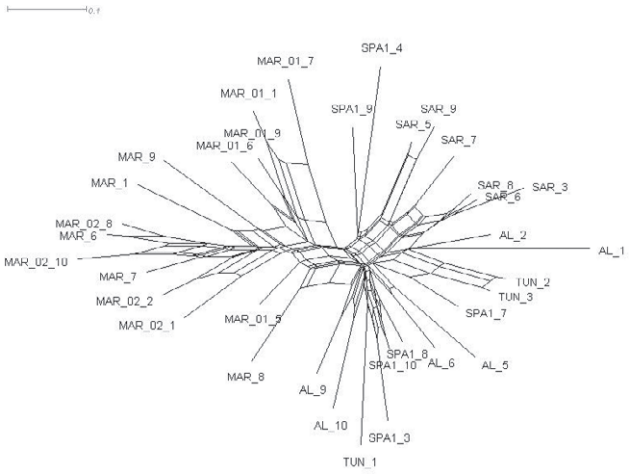

Figure. 5. Split tree of 35 *Chamaerops humilis* genotypes of five putative populations based on Nei and Li's genetic distance. MAR: Terketen, Morocco; MAR1: Beni Mellal, Morocco; MAR2: Touama, Morocco; SPA: Valencia, Spain; ALG: Sidi Belattar, Algeria; TUN: Cap Serrat, Tunisia; SAR: Porto Tangone, Sardinia, Italy.

number of observed alleles per locus (16) while locus16 and locus23 the lowest (3); the average number of alleles per locus was 7.1. Seven SSR markers were found highly informative (PIC > 0.5) and three reasonably informative (0.25 < PIC < 0.5); PIC average was 0.62.

For eight SSR loci the expected heterozygosity (He) was higher than the observed heterozygosity, except for locus35 and locus44, which deviated from Hardy Weinberg equilibrium. Mean He resulted 0.67 and the mean Ho was 0.54.

Interestingly, a rare allele of 97 bp at the locus37 was found restricted to two genotypes from Terketen (MAR1 and MAR8). Neighbor-Net method cluster analysis (Figure 5) showed that the Moroccan genotypes separated from all genotypes of the other geographical areas. Overall genotypes from Beni Mellal presented an intermediate genetic proximity with other populations. Two genotypes MAR6 and MAR7 from Terketen showed closer relationship with Touama genotypes. Algerian, Sardinian, Spanish, and Tunisian genotypes shared closer relationships and genotypes from Sardinia grouped together.

DISCUSSION

The large intrapopulational morphological variation observed testifies a large genetic variability among the studied populations. This variation was proven by the molecular analysis.

A rich assortment of SSR alleles was found indicating a greater genetic diversity than that previously identified on 705 Sicilian dwarf palm genotypes using 28 SSR markers (Giovino *et al.* 2014). This information is useful for acquiring new knowledge on the species and for planning a more extensive work on the whole area of distribution of this species in order to acquire a more detailed knowledge to preserve *Chamaerops humilis* genetic diversity in the future.

Table 2. SSR locus name, number of alleles (No), observed (Ho) and expected heterozygosities(He), polymorphic information content (PIC) of 10 microsatellite loci in a sample of 35 accessions of *Chamaerops humilis*.

Locus name	Allele No	H exp	Ho	PIC
locus19	6	0,64	0,36	0,55
locus25	12	0,84	0,44	0,8
locus27	9	0,85	0,47	0,82
locus15	5	0,63	0,58	0,57
locus16	3	0,5	0,36	0,43
locus23	3	0,26	0,14	0,25
Locus35	6	0,65	0,83	0,58
Locus37	16	0,9	0,72	0,88
Locus44	4	0,58	0,97	0,49
Locus48	7	0,83	0,55	0,79
Mean	7,1	0,668	0,542	0,62

As concern the presence of a rare allele restricted to some genotypes of the Terketen population, it is possible to speculate that this allele may reflect an adaptation to particular environment conditions or stresses. It has been shown in many species that SSR diversity is adaptive, influenced by natural and anthropic selection and correlated with ecological-edaphic and genetic factors (Marchese *et al.* 2010, Marchese *et al.* 2017). Natural selection plays an essential role in controlling the length of a repeat (Li *et al.* 2000). King and Soller (1999) proposed that changes in length of SSRs functionally integrated into the genome can influence plant adaptive fitness. However, further molecular studies are needed, including a greater number of genotypes representative of all dwarf palm populations, to confirm the uniqueness of this allele.

In the Neighbor-Net cluster analysis the Moroccan genotypes grouped together and appeared separated from Algerian, Sardinian, Spanish, and Tunisian genotypes which seemed to share closer relationships, among this latter group the Sardinian genotypes grouped jointly. In general, the Neighbor-Net cluster analysis was in agreement with the discriminant analysis.

According to the observed variability, it seems opportune to distinguish the populations of high altitude of Atlas and Anti-Atlas as a distinct subspecies.

A careful bibliographic research has allowed us to ascertain that the name *Chamaerops humilis* var.

argentea André Rev. Hort. 57: 231 (1885) quoted by the large part of repertoires and floras (e.g. Maire 1957, Fennane 2014) has never been published either in that place or, as far as we have been able to verify, in other sources. So, the first validly published name that can refer to this entity is *C. humilis* var. *cerifera* Becc. described on material cultivated in Naples of dubious origin. The study of original material housed in FI, where the Herbarium by Beccari is housed (Cuccuini and Nepi 2006) was not sufficient to dispel the doubts because the single specimen found (Beccari n. 384) consists only of badly preserved fruits. In any case the only known populations in nature with grey leaves, due to the high concentration of waxes on their surface, are found exclusively in Morocco at high altitudes, thus the taxon described by Beccari, by reasonable assumption, refers to these populations.

The following new combination is proposed:

Chamaerops humilis subsp. ***cerifera*** (Becc.) Giovino & Domina subsp. nov. (≡*C. humilis* var. *cerifera* Becc. in Webbia 5(1): 65 1921).

Type: Lectotype (here designated): Beccari n. 384, *Chamaerops humilis* L. v. *cerifera* Becc.; Italia, Napoli, n. 1919, Ruffo, ex Ruffo principe di S. Antimo (FI).

This subspecies differs from *C. humilis* subsp. *humilis* by its leaves glaucous-silvery, dull, covered with persistent scaly hairs. The individuals in form of compact tufts with short stems and smaller leaves blades commonly found in high altitude populations in High Atlas and Anti Atlas are due to the effect of environmental and anthropozoogenic degradation.

CONCLUSION

The obtained results agree with those by García-Castaño *et al.* (2014): *C. humilis* has a large morphological and genetic variation throughout its distribution range. The southern populations from Morocco are isolated from the resting ones and in particular high-altitude populations are well distinct from the morphological and genetic points of view due to a speciation underway moved by ecological and spatial separation to which they are subjected. Thus, it is here proposed to discriminate these populations within a separated subspecies.

These results encourage about the possibility of cultivating *Chamaerops humilis* subsp. *cerifera* also in environments with lower temperatures than the Mediterranean coasts where *C. humilis* subsp. *humilis* has been confined so far. Such cultivations would have primary interest as ornamental but the possibility of extraction of medicinal principles is not excluded.

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Supplemental Data file 1. Mean of the measures on morphological characters used for statistical analysis.

Code	Population	Height of the stem (cm)	Height of the plant (cm)	Crown diameter (cm)	Stem diameter (mm)	Petiole width (cm)	Petiole width (cm)	Leaf length (cm)	Leaf width (cm)	Leaf thickness (mm)	No. of leaf segments	No. thorns on the petiole	Wax coverage density %
MAR_1	Terketen, Morocco	7,5	17	27	34,5	3,9	3,2	8,7	11,8	1	9	0	95
MAR_2	Terketen, Morocco	8	20	22	29,9	3,1	3,5	9,7	13,7	0,8	8	5	95
MAR_3	Terketen, Morocco	6	20	28	28,6	0,9	4,1	11,5	14	0,8	9	8	95
MAR_4	Terketen, Morocco	4,5	15,5	19	34,5	0,8	3,3	9,5	11	0,7	10	1	95
MAR_5	Terketen, Morocco	9	19	24	47,5	3,3	2,7	8,8	10,6	0,5	9	2	95
MAR_6	Terketen, Morocco	6	20	21	29,4	4,2	4,2	12,7	15,5	1,1	9	0	95
MAR_7	Terketen, Morocco	6	26,5	31	23,8	3,7	6	13,6	17,7	0,7	11	4	95
MAR_8	Terketen, Morocco	8	20	23	31,5	3	4,7	10,3	13	0,9	11	0	95
MAR_9	Terketen, Morocco	11,5	29	36	34,5	3,4	6	11,3	15	0,8	11	6	95
MAR_10	Terketen, Morocco	9,5	22	24	20,5	3,2	3,7	9,4	10,5	0,5	8	9	95
MAR_11	Terketen, Morocco	7	18,5	26	34,9	3,3	5	9	11,5	1,2	9	10	95
MAR_12	Terketen, Morocco	7,5	23	30,5	34,9	3,9	4,5	10,2	13,2	0,9	11	7	95
MAR_13	Terketen, Morocco	8	21,5	28	27,3	2,7	3,2	9	10,5	0,4	9	4	95
MAR_14	Terketen, Morocco	6	16	20	31,7	3,2	2,7	5,2	10,7	0,7	9	4	95
MAR_15	Terketen, Morocco	8	20	21	29,5	2,9	2,8	11,4	13,7	0,8	10	5	95
MAR_16	Terketen, Morocco	7,5	23	24	26,2	3,1	2,7	10	10,2	0,4	9	11	95
MAR_17	Terketen, Morocco	8	22,5	26,5	34,8	2,5	4,4	10,7	10,1	0,5	9	1	95
MAR_18	Terketen, Morocco	9	21	29,2	34,2	3,1	4,3	9,7	8,5	0,7	8	3	95
MAR_19	Terketen, Morocco	4	12	16	33,5	2,8	2	6,5	9,5	0,5	7	15	95
MAR_20	Terketen, Morocco	8	21	32	34,4	3,9	4,5	9,8	12,2	1	10	5	95
MAR_21	Terketen, Morocco	6,5	27,5	32	33,4	3	9,5	14,3	19,2	0,5	9	9	95
MAR_22	Terketen, Morocco	8	21,5	30	38,5	3,1	4,2	9,5	10,2	0,4	9	13	95
MAR_23	Terketen, Morocco	10	25	31	28,2	3	5,5	12,3	14,5	0,5	11	12	95
MAR_24	Terketen, Morocco	6	2,6	30,5	35,4	3	4,5	10,5	13,2	0,5	10	12	95
MAR_25	Terketen, Morocco	10	23,5	35,5	44,8	2,6	4,7	10	14,8	0,5	10	6	95
MAR_26	Terketen, Morocco	4	21	23	27,8	3	3,8	9,7	13,2	0,6	9	1	95
MAR_27	Terketen, Morocco	6	20	29,5	30,1	3,5	4,8	10,2	13,8	0,6	9	11	95
MAR_28	Terketen, Morocco	7	21	29	24,1	3,1	3,7	9,5	14	0,6	9	7	95
MAR_29	Terketen, Morocco	4	20	30	24,4	2,7	5	12,3	16,8	0,6	8	4	95
MAR_30	Terketen, Morocco	9	22	28,5	34,2	3,4	5,3	12	15,2	0,8	11	7	95
MAR_31	Terketen, Morocco	6	24	34,5	29,8	3,3	6,3	12,3	14,2	0,8	8	11	95
MAR1_1	Beni Mellal, Morocco	4	20	30	14,3	3,8	2	16,8	11	0,9	4	5	55
MAR1_2	Beni Mellal, Morocco	4	17	31	26,5	4,6	4	15	14	0,8	10	6	55
MAR1_3	Beni Mellal, Morocco	3,5	17,5	29	4,3	3,7	2,3	16,7	12,7	0,7	5	3	55
MAR1_4	Beni Mellal, Morocco	4	15	20	16,4	4	2,2	12	12,3	1	7	5	65
MAR1_5	Beni Mellal, Morocco	3	15,5	17	16	3,3	2,7	10,3	9	0,6	6	5	65
MAR1_6	Beni Mellal, Morocco	4	14	17	20	3,5	2	10,2	9,3	1,3	7	3	65
MAR1_7	Beni Mellal, Morocco	4	14	17	20	2,5	1,5	9	6	0,6	6	0	65
MAR1_8	Beni Mellal, Morocco	3,5	21,5	41	20	3,4	5,3	16,3	12	0,7	6	6	55
MAR1_9	Beni Mellal, Morocco	4	14,5	23	24	1,2	3,2	11,7	8	0,8	5	0	55
MAR1_10	Beni Mellal, Morocco	5	18,5	25	18,3	3,5	3,2	15,2	12,3	0,8	11	5	65
MAR1_11	Beni Mellal, Morocco	2	12,5	21	17	3,3	2,8	12,2	8	0,7	7	3	75
MAR1_12	Beni Mellal, Morocco	3	18	18	23	3,9	2,2	13,7	9	0,9	6	3	65
MAR1_13	Beni Mellal, Morocco	3	19	25	18	3,8	3	14,5	9	0,7	6	6	75
MAR1_14	Beni Mellal, Morocco	4	16,5	24	19	3,5	2,9	12,6	9	0,9	6	3	55
MAR1_15	Beni Mellal, Morocco	4	17,5	23	17,5	3,3	2,6	11	9,3	0,9	5	3	55

Code	Population	Height of the stem (cm)	Height of the plant (cm)	Crown diameter (cm)	Stem diameter (mm)	Petiole width (cm)	Petiole width (cm)	Leaf length (cm)	Leaf width (cm)	Leaf thickness (mm)	No. of leaf segments	No. thorns on the petiole	Wax coverage density %
MAR1_16	Beni Mellal, Morocco	3	18	22	23	3,6	2,9	13	9,2	0,7	7	5	65
MAR1_17	Beni Mellal, Morocco	3	16,5	20	17	3,8	3,2	12,8	9,2	0,7	6	3	65
MAR1_18	Beni Mellal, Morocco	3,5	16	24	23	3,7	2,9	12,5	8,7	0,9	6	3	76
MAR1_19	Beni Mellal, Morocco	3	17	23	23	2,9	3,2	16	12,5	0,7	6	5	65
MAR1_20	Beni Mellal, Morocco	4	15	26	21	3,6	4	16	13,5	0,9	6	3	55
MAR1_21	Beni Mellal, Morocco	4	15,5	25	18	3,5	3,6	17	13,2	0,6	7	3	65
MAR1_22	Beni Mellal, Morocco	3,5	16,5	19	23	3,2	2,8	12,7	8,3	0,7	6	5	76
MAR1_23	Beni Mellal, Morocco	3	18	25	18,5	3	3,1	15,1	12	0,9	6	3	65
MAR1_24	Beni Mellal, Morocco	5	19	24	19	2,9	2,7	12	9,8	0,9	6	5	65
MAR1_25	Beni Mellal, Morocco	4	18	19	19	3,3	3,5	16,6	13,2	0,6	7	0	55
MAR1_26	Beni Mellal, Morocco	3,5	17,5	23	18	3,5	3,4	17,2	13	0,7	6	5	65
MAR1_27	Beni Mellal, Morocco	4	18	24	18,5	3,6	3,3	12	8,2	0,9	5	3	65
MAR1_28	Beni Mellal, Morocco	3,5	18,5	22	21	3	4	15,5	13,8	0,7	6	5	75
MAR1_29	Beni Mellal, Morocco	4	17	20	22	2,9	2,8	14	12,6	0,9	6	3	55
MAR1_30	Beni Mellal, Morocco	3	18	24	23	3,4	3	13,7	8,9	0,8	7	3	65
MAR1_31	Beni Mellal, Morocco	4	17	24	26	3,6	2,7	12	10,3	1,1	7	3	55
MAR2_1	Touama, Morocco	3	17,5	25	20	3,4	3,5	14,3	10,7	0,4	7	0	55
MAR2_2	Touama, Morocco	3	13	15	19	3,1	2,2	10,5	8	0,4	7	0	65
MAR2_3	Touama, Morocco	3	16	16	22	2,8	2,3	12,7	10,7	0,7	6	0	75
MAR2_4	Touama, Morocco	3	15	18	16	3,6	2,7	11,7	12,7	0,7	9	0	75
MAR2_5	Touama, Morocco	4	14	20	12	3,6	2,7	10,3	10	0,9	6	0	75
MAR2_6	Touama, Morocco	3	17	23	12	2,6	5,3	13,7	10	0,7	7	0	65
MAR2_7	Touama, Morocco	5	14	10	25	2,3	3	8,7	6	1,4	5	0	55
MAR2_8	Touama, Morocco	4	16	22	20	2,6	3	12,6	10,8	0,6	6	0	55
MAR2_9	Touama, Morocco	4	17	23	22	2,7	3,1	13,5	10	0,7	5	0	65
MAR2_10	Touama, Morocco	3	17	18	18	2,6	2,8	10,5	9,6	0,9	7	0	55
MAR2_11	Touama, Morocco	3	18	22	20	3	2,9	11,9	12,5	0,6	6	0	65
MAR2_12	Touama, Morocco	5	16	23	22	2,6	4	12,7	10,7	0,7	5	0	75
MAR2_13	Touama, Morocco	4	16	18	20	2,8	2,8	12	10	1	7	0	55
MAR2_14	Touama, Morocco	3	17	20	18	2,8	2,7	10,7	9,8	0,6	5	0	65
MAR2_15	Touama, Morocco	3	17	23	22	3	3	13,5	9,5	0,7	6	0	55
MAR2_16	Touama, Morocco	5	18	20	18	4	2,8	11,9	9	0,9	7	0	75
MAR2_17	Touama, Morocco	5	17	18	20	2,8	3	12,7	10,5	1	6	0	65
MAR2_18	Touama, Morocco	4	16	22	25	2,8	3	10,8	9,8	0,6	5	0	75
MAR2_19	Touama, Morocco	4	17,5	23	23	3,1	2,7	12,2	9,6	0,7	6	0	55
MAR2_20	Touama, Morocco	3	18	22	22	3	2,6	13,1	8,7	1	7	0	65
MAR2_21	Touama, Morocco	3	17	20	19	2,8	2,9	12,7	10,7	0,9	6	0	75
MAR2_22	Touama, Morocco	3	18	20	21	4	3,1	12,2	10,5	0,6	6	0	55
MAR2_23	Touama, Morocco	4	17,5	22	20	2,6	2,5	13,4	9	0,7	7	0	65
MAR2_24	Touama, Morocco	5	16	23	20	3	2,6	9,6	7	0,9	6	0	75
MAR2_25	Touama, Morocco	4	16	22	18	2,8	2,7	12,4	10,7	0,6	6	0	55
MAR2_26	Touama, Morocco	3	14	24	20	3,2	2,8	11,3	10,3	0,6	5	0	75
MAR2_27	Touama, Morocco	3	15	21	22	3,2	4,2	13,7	10	0,7	6	0	55
MAR2_28	Touama, Morocco	5	15,5	20	19	2,8	3,2	15	11,4	0,7	7	0	75
MAR2_29	Touama, Morocco	4	17,5	20	21	2,9	3,6	15,3	10,5	0,6	6	0	55
MAR2_30	Touama, Morocco	4	17	22	22	3	3,6	12,8	10,5	0,9	7	0	55
MAR2_31	Touama, Morocco	3	17	18	19	2,6	3	13	11,7	0,7	7	0	65

Code	Population	Height of the stem (cm)	Height of the plant (cm)	Crown diameter (cm)	Stem diameter (mm)	Petiole width (cm)	Petiole width (cm)	Leaf length (cm)	Leaf width (cm)	Leaf thickness (mm)	No. of leaf segments	No. thorns on the petiole	Wax coverage density %
ALG1	Sidi Belattar, Algeria	7,5	29	31	19,8	3,6	7,3	21,3	18,3	0,7	8	8	25
ALG2	Sidi Belattar, Algeria	7	25,5	41	25,1	3,8	6,2	21,7	19,7	0,6	10	8	25
ALG3	Sidi Belattar, Algeria	7	23	27	19,8	3,9	5	15	16	0,9	7	8	25
ALG4	Sidi Belattar, Algeria	6,5	22,5	24,5	21	3,7	6	18,3	14,2	0,8	5	6	25
ALG5	Sidi Belattar, Algeria	6	26	32,5	24	3,3	7,2	21,5	15,2	0,6	6	6	25
ALG6	Sidi Belattar, Algeria	2,5	25,5	35	14,3	3,8	6,7	24,2	8,3	0,4	4	7	25
ALG7	Sidi Belattar, Algeria	3,5	21	29	12	3,9	5,2	20,7	12,2	0,8	6	7	25
ALG8	Sidi Belattar, Algeria	4	22	37	19,2	4,2	6,2	20,7	11,3	0,8	7	7	25
ALG9	Sidi Belattar, Algeria	4	18	31	12,3	3,8	2,2	16	7,3	0,9	4	2	30
ALG10	Sidi Belattar, Algeria	7	28	41	24,6	3,2	6	22	10,3	0,8	6	7	35
ALG11	Sidi Belattar, Algeria	5	18	27	13,8	3,4	4	14,5	8,7	0,5	7	6	35
ALG12	Sidi Belattar, Algeria	5	20	26	29,1	3,1	10	3,8	6,8	0,5	10	4	35
ALG13	Sidi Belattar, Algeria	7	18	28	21,7	3,7	5,2	19,2	5,3	0,8	6	5	45
ALG14	Sidi Belattar, Algeria	8	29,5	34	35,4	3,6	7,7	19,3	13,7	0,7	12	13	45
ALG15	Sidi Belattar, Algeria	3	22	38	19,5	3,9	4,8	8,7	8,3	0,9	7	8	45
ALG16	Sidi Belattar, Algeria	6	30	33	14,8	3,1	5,8	20,3	7,3	0,7	4	5	45
ALG17	Sidi Belattar, Algeria	5	23	30	23	3,2	4,7	15	10,7	0,6	7	1	45
ALG18	Sidi Belattar, Algeria	5	21	29	34,3	3,1	5,5	15,8	11,7	1,3	7	5	45
ALG19	Sidi Belattar, Algeria	7	16	26,5	26	4	3,8	12,7	7	0,6	9	4	45
ALG20	Sidi Belattar, Algeria	5,5	27,5	30	14,5	3,5	5,2	21	7,2	0,6	7	6	45
ALG21	Sidi Belattar, Algeria	4	20	30	25	3,9	4,5	16	9,8	0,8	7	2	45
ALG22	Sidi Belattar, Algeria	4,5	20	28	24,8	3,6	6	17,2	14,8	1	8	4	45
ALG23	Sidi Belattar, Algeria	5,5	28,5	33,5	17,3	3,5	6,2	22	12,3	1	5	6	45
ALG24	Sidi Belattar, Algeria	4	22	30	12,4	4	6,5	20	6	0,8	7	9	45
ALG25	Sidi Belattar, Algeria	5	28	28	15,7	3,7	6,2	18,2	9,7	0,9	6	5	45
ALG26	Sidi Belattar, Algeria	7	23	39	25,8	4,3	5,3	21,8	14,3	0,7	8	8	35
ALG27	Sidi Belattar, Algeria	4	21	24	31	4,4	3,3	14,5	8	1,2	6	4	35
ALG28	Sidi Belattar, Algeria	4	26	31	16,2	5,2	2,7	17	16	0,7	9	3	35
ALG29	Sidi Belattar, Algeria	6	20	25	20,5	4,1	3,7	11,8	11,7	1,1	10	3	35
ALG30	Sidi Belattar, Algeria	5	25	29	15,8	3,8	4,5	19,7	8	0,8	8	3	35
ALG31	Sidi Belattar, Algeria	6	21	36	20,3	3,6	5,7	17,2	12,8	0,5	6	7	35
SPA1	Valencia, Spain	6,5	24	27,5	23,6	4,1	5,2	16	16,2	1,6	9	7	35
SPA2	Valencia, Spain	4	28	29,5	15,3	3,7	7,2	23	24,5	0,9	6	11	35
SPA3	Valencia, Spain	3,5	30	34	13,1	3,5	5,7	29	19,3	1,2	6	7	35
SPA4	Valencia, Spain	3	21	24	16,9	4,3	3,3	16	18	2,8	6	5	35
SPA5	Valencia, Spain	6	24	32	18,8	4,4	4,3	19,5	15	1,8	4	7	35
SPA6	Valencia, Spain	5	20,5	24	18,4	3,1	6,7	19,2	18,2	2	7	8	35
SPA7	Valencia, Spain	2,5	20,5	24	13,5	4,1	3,5	14,7	12,8	0,9	5	5	35
SPA8	Valencia, Spain	4	27	27	14,2	4,4	4,8	23,7	9,7	1,9	4	4	30
SPA9	Valencia, Spain	6	29,5	25	29,3	3,8	4,7	19,2	15,8	1,3	5	7	25
SPA10	Valencia, Spain	3,5	27	30	19,3	3,4	7	19,7	16	2	6	9	25
SPA11	Valencia, Spain	4,5	26	29,3	18,5	3,8	7,8	22,2	15	1,5	7	11	25
SPA12	Valencia, Spain	4	24,5	30	23	3,8	7,5	20,7	16	1,3	7	10	25
SP13	Valencia, Spain	5,5	28,5	24	21,7	4,7	5,7	5,8	14	1,6	7	11	25
SPA14	Valencia, Spain	4	35	39	33,8	6,2	7,8	26	24	2,3	9	11	25
SPA15	Valencia, Spain	4	23	26	21,7	2,9	4,3	17	10	1,4	7	7	35
SPA16	Valencia, Spain	4	27,5	33	20,1	4,3	6,5	19	15,8	1,7	7	10	35

Code	Population	Height of the stem (cm)	Height of the plant (cm)	Crown diameter (cm)	Stem diameter (mm)	Petiole width (cm)	Petiole width (cm)	Leaf length (cm)	Leaf width (cm)	Leaf thickness (mm)	No. of leaf segments	No. thorns on the petiole	Wax coverage density %
SPA17	Valencia, Spain	4,5	27	20,5	22,2	4,2	4,7	17,5	14,7	0,9	8	6	35
SPA18	Valencia, Spain	4	21	28,5	22,3	4,9	4,5	17,7	20,7	1,6	8	11	35
SPA19	Valencia, Spain	3,5	21,5	27	22,5	4,3	4,7	15,5	14,3	1,6	7	7	35
SPA20	Valencia, Spain	2,5	20,5	24	13,2	4,1	2,2	16	11,7	1,4	6	3	35
SPA21	Valencia, Spain	3,5	20	24,5	15,4	5,4	2,3	15,7	12	1,1	6	3	35
SPA22	Valencia, Spain	3,5	18	23	14,5	5,1	2,5	13,5	13	1,3	7	2	35
SPA23	Valencia, Spain	5	22,5	26	17,3	3,9	2,8	19,2	14,5	1,3	6	8	35
SPA24	Valencia, Spain	5	28	28	19,1	4,2	7	22,3	14,3	1,4	7	11	35
SPA25	Valencia, Spain	3	30	29,5	18	5,4	4,5	23,2	14,2	1,7	6	3	35
SPA26	Valencia, Spain	4	33	36	12,6	4,3	6,7	27,7	22,5	2,5	6	10	35
SPA27	Valencia, Spain	4	25,5	26	17	4,5	5,5	20	11,8	1,5	6	3	35
SPA28	Valencia, Spain	3	28	23	11	3,7	5,7	19,5	13,2	0,9	7	8	35
SPA29	Valencia, Spain	3,5	26,5	21	12,8	4,1	6	16,3	11,8	0,9	7	6	35
SPA30	Valencia, Spain	4	27	25,5	13,4	3,8	5,8	13,3	12,2	0,7	6	10	35
SPA31	Valencia, Spain	3	27,5	22	11,5	3,4	5,5	12,5	12,2	1	5	7	35
TUN1	Cap Serrat, Tunisia	3	31	33,5	20,3	4,2	8,3	27,3	23	0,8	7	8	25
TUN2	Cap Serrat, Tunisia	4	41	46	33,5	4,3	11,7	30	20	0,6	7	11	25
TUN3	Cap Serrat, Tunisia	2	24.5	22	17,7	3,6	2,3	16,3	11,8	1,2	4	0	25
TUN4	Cap Serrat, Tunisia	2	16	26	18,1	3,7	3	13	11,3	1,3	7	0	25
TUN5	Cap Serrat, Tunisia	2	18	28	20,6	4,7	3,3	16,3	16,3	0,8	10	5	25
TUN6	Cap Serrat, Tunisia	6	36,5	43	20,2	4,3	5,8	22,2	22	0,9	9	11	25
TUN7	Cap Serrat, Tunisia	5	28,5	32	22,8	3,9	6,7	20,3	10	0,6	8	7	25
TUN8	Cap Serrat, Tunisia	7	37,5	38	27,5	5	11,3	25,8	25,7	0,7	10	10	25
TUN9	Cap Serrat, Tunisia	4,5	27,5	34	18	5,1	8,8	24,8	15,7	1	5	5	25
TUN10	Cap Serrat, Tunisia	6,5	35	38	36	5,9	10,5	24,2	17,7	1	11	11	25
TUN11	Cap Serrat, Tunisia	5	30,5	40	22	4,3	8,2	26,8	19	0,5	9	7	25
TUN12	Cap Serrat, Tunisia	5	22	35	16,4	3,8	3,2	17,8	13,7	0,7	5	1	25
TUN13	Cap Serrat, Tunisia	3	23	37	16,7	3,7	5,2	21,3	8	0,7	3	0	25
TUN14	Cap Serrat, Tunisia	6	32	41,5	28,8	4,9	8,3	24,5	18	0,8	9	8	25
TUN15	Cap Serrat, Tunisia	6,5	33	34	20	4,2	6,5	24,2	12	0,7	4	5	25
TUN16	Cap Serrat, Tunisia	6	26,5	26	20,1	3,9	4,2	18,2	11,7	0,6	5	3	25
TUN17	Cap Serrat, Tunisia	6,5	47	56	32,3	4,8	12,7	31	23,7	1,7	12	10	25
TUN18	Cap Serrat, Tunisia	10	43	48	42	5,3	11,3	33,3	21,3	1	8	9	25
TUN19	Cap Serrat, Tunisia	7	38	41	28,3	4,7	7,7	22,7	15	0,7	6	10	25
TUN20	Cap Serrat, Tunisia	9	46,5	50	47	5,4	15,8	31,7	23	0,9	10	11	25
TUN21	Cap Serrat, Tunisia	7	44	45	38,3	4,6	12	25,8	22,3	1	10	12	25
TUN22	Cap Serrat, Tunisia	11	54,5	60	42,5	5,6	18	38,3	20,7	0,7	12	11	25
TUN23	Cap Serrat, Tunisia	7,5	38	43	35	4,8	8,7	16,3	25	1,1	10	7	25
TUN24	Cap Serrat, Tunisia	8	49,5	45	38	6	12,7	38,3	14	1,1	8	7	25
TUN25	Cap Serrat, Tunisia	6	41	49	28,1	5,2	12,8	31,8	13	1,5	5	9	25
TUN26	Cap Serrat, Tunisia	7	30	33	30	4,2	6,7	19,3	15,7	1,1	6	7	25
TUN27	Cap Serrat, Tunisia	7,5	38	44	44,2	5,1	12	27,3	10	1,1	9	7	25
TUN28	Cap Serrat, Tunisia	5,5	35	37	23	4,4	7,8	26,2	15	0,7	4	8	25
TUN29	Cap Serrat, Tunisia	7	38,5	50	34,4	6	14,8	32,2	20	1,4	12	12	25
TUN30	Cap Serrat, Tunisia	5	31	40	19	4,4	5,7	23	12	1,3	6	9	25
TUN31	Cap Serrat, Tunisia	3	27	34	16	3,4	5,5	24	11	1	3	3	25
SAR1	Porto Tangone, Sardinia	10	29,5	30	16	4,8	4,3	14	16,5	3,7	8	6	25

Code	Population	Height of the stem (cm)	Height of the plant (cm)	Crown diameter (cm)	Stem diameter (mm)	Petiole width (cm)	Petiole width (cm)	Leaf length (cm)	Leaf width (cm)	Leaf thickness (mm)	No. of leaf segments	No. thorns on the petiole	Wax coverage density %
SAR2	Porto Tangone, Sardinia	3	19	22	11,5	3,1	2,7	12,5	9,5	2	4	1	25
SAR3	Porto Tangone, Sardinia	4,5	20,5	24	16,8	4,5	2,8	14,8	15,2	5	9	7	25
SAR4	Porto Tangone, Sardinia	4	22	12,5	12,5	4	1,3	17,7	9	4,3	5	0	35
SAR5	Porto Tangone, Sardinia	2,5	18,5	14	9,8	3,9	1	10,8	8,3	3,4	4	0	35
SAR6	Porto Tangone, Sardinia	5,5	21	21,5	12,7	3,6	2,3	12,8	10,2	5,7	5	5	35
SAR7	Porto Tangone, Sardinia	3,5	21	27,5	12,9	3,4	2,7	13,7	15,7	4,7	8	3	35
SAR8	Porto Tangone, Sardinia	3,5	20,5	26,5	14,8	3,9	2,7	15,5	15,8	9,3	5	0	30
SAR9	Porto Tangone, Sardinia	5	26	23	15,9	3	4	16	13,7	3,2	3	5	25
SAR10	Porto Tangone, Sardinia	4	26	27,5	23,8	3,9	4,5	16,7	17,3	4,3	11	5	25
SAR11	Porto Tangone, Sardinia	3	22,5	29,5	10,6	3	3,3	16,3	13,3	3,5	5	3	30
SAR12	Porto Tangone, Sardinia	4,5	28	26	18,5	4,8	5,3	19,8	17	3,7	8	7	35
SAR13	Porto Tangone, Sardinia	5	25	26,5	17	3,8	3,8	20	12,5	4,7	8	5	30
SAR14	Porto Tangone, Sardinia	4	32	27,5	9,9	4,5	4,5	22	13,7	4,5	5	3	35
SAR15	Porto Tangone, Sardinia	4,5	30	27,5	16,9	4,7	4,2	21,3	13	5,6	3	5	25
SAR16	Porto Tangone, Sardinia	6	27	23	22	3,8	3,6	18,9	14,3	4,7	5	3	25
SAR17	Porto Tangone, Sardinia	5	25	27,5	21,8	3,9	4,1	19,1	15	4,3	4	0	30
SAR18	Porto Tangone, Sardinia	4,5	19	26,5	10,7	4,5	4,5	22	13,6	4,7	8	5	30
SAR19	Porto Tangone, Sardinia	4	32	27,5	16,7	4,7	4,1	20,6	14,7	5,5	5	3	35
SAR20	Porto Tangone, Sardinia	6	22	27,5	10,4	3,8	3,8	17,3	11,6	4,7	5	3	40
SAR21	Porto Tangone, Sardinia	5	27	23	18,4	4	4,2	14,1	8,9	4	4	0	30
SAR22	Porto Tangone, Sardinia	4,5	25	27,5	16,9	4,5	4,5	16,3	15,4	5,6	5	3	25
SAR23	Porto Tangone, Sardinia	4	19	26,5	23	4,3	3,8	22,2	13,4	4,5	4	5	30
SAR24	Porto Tangone, Sardinia	6	31	19	17,5	3,8	3,6	19,5	16,2	4,7	5	5	35
SAR25	Porto Tangone, Sardinia	4	22	27,5	16,9	4,2	4,5	22,6	15,3	4,3	3	3	25
SAR26	Porto Tangone, Sardinia	4,5	24	26,5	20,3	4,5	4,2	19,4	16,3	4	4	0	30
SAR27	Porto Tangone, Sardinia	5	28	27,5	18,6	3,9	3,8	19,2	16,2	4,7	8	5	35
SAR28	Porto Tangone, Sardinia	3,5	31	23	17,5	3,8	3,6	22	14	3,8	5	0	30
SAR29	Porto Tangone, Sardinia	6	24	26,5	18,3	4,5	4,5	21,6	15,8	4,1	4	3	25
SAR30	Porto Tangone, Sardinia	5,5	28	23	17,4	4,1	4,1	23	15,8	4,6	5	5	30
SAR31	Porto Tangone, Sardinia	7	22	23	15,8	3,9	3,7	9,8	13,3	3,4	8	4	35

Supplemental data file 2. Pearson correlation coefficients (r) among the 12 characters measured. All correlations were significant with p > 0.0001 (Student's t test) with the lone exception of Leaf thikness.

	H stem	H plant	Crown diameter	Stem diameter	Petiole width	Petiole length	Leaf length	Leaf width	Leaf thikness	No. leaf segments	No. thorns	Hair density
H stem		3,42E-08	1,82E-08	3,39E-23	0.048467	1,14E-07	0.079418	1,98E-01	0.24308	7,31E-13	3,29E-06	0.0058902
H plant	0.4303		1,55E-41	7,09E-02	4,91E-22	9,76E-52	2,97E-45	1,70E-21	0.0277	0.0065374	1,04E-14	3,97E-13
Crown diameter	0.45465	0.77937		1,41E-06	7,02E-14	6,31E-52	3,46E-31	2,11E-15	0.18042	1,34E-02	2,91E-18	1,30E-04
Stem diameter	0.64769	0.29956	0.4178		0.3676	7,78E-10	0.44561	0.0001304	4,70E-02	3,28E-20	2,50E-03	9,37E-03
Petiole width	0.13412	0.63638	0.54079	0.061459		3,93E-12	9,67E-23	9,64E-12	6,58E-01	0.14084	5,07E-06	9,39E-19
Petiole length	0.4396	0.82722	0.82799	0.47882	0.51553		4,07E-33	8,69E-17	0.082782	4,18E-04	2,50E-19	7,00E-05
Leaf length	0.11934	0.79836	0.71441	0.052045	0.64331	0.72809		5,46E-16	0.013342	0.39458	3,73E-06	1,16E-26
Leaf width	0.28525	0.63096	0.56117	0.25682	0.49385	0.57859	0.55578		0.0043571	6,97E-04	1,80E-12	5,84E-03
Leaf thikness	-0.079577	0.14947	-0.091265	-0.30507	0.26751	-0.11803	0.16776	0.19284		5,25E-01	0.14078	2,41E-05
No. leaf segments	0.52635	0.1841	0.32128	0.61759	0.1003	0.36163	-0.058079	0.35603	-0.27094		2,85E-05	1,18E-02
No. thorns	0.41007	0.53844	0.59605	0.34151	0.38371	0.60805	0.40892	0.52061	-0.10031	0.38944		0.0071865
Hair density	0.18638	-0.53018	-0.37405	0.32569	-0.60164	-0.38043	-0.67882	-0.33146	-0.39111	0.32287	-0.182	

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Genotoxicity testing of bovine lymphocytes exposed to epoxiconazole using alkaline and neutral comet assay

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Abstract. Epoxiconazole belongs in the class of azoles which have been developed to protect crops from fungal diseases. The mechanism of action of these fungicides is to inhibit the specific cytochrome P450 enzyme (CYP), CYP51 (lanosterol 14α-demethylase) which contributes to ergosterol biosynthesis. Since ruminants and cattle are exposed to contaminants during grazing, they are a suitable experimental model for genotoxicity testing. In our experiment, epoxiconazole (EPX) (active agent, 99% of purity) was tested *in vitro* for its potential genotoxic and cytotoxic effects on bovine lymphocytes, isolated from whole peripheral blood. We exposed the lymphocytes to EPX at concentrations of 2.5, 5, 10, 25, 50 and 100 μg/mL by two different ways: immediately after isolation of lymphocytes during 2 h in RPMI 1640 medium (without phytohaemagglutinin, PHA) as well as on the last 2 h of 48-h culture (with PHA). In a second case, we chose 48 h culture because the lymphocytes usually start DNA replication 24 h after the start of the cultures; therefore, we incubated the cells longer to obtain dividing (proliferating) cells. The levels of DNA damage were measured using alkaline and neutral comet assays. The results of alkaline comet assay showed the significantly increased percentage of DNA breaks in both lymphocytes in medium without PHA (2 h of exposure; non-proliferating cells) and lymphocytes cultured during 48 h in medium with PHA (exposure for the last 2 h of cultivation; proliferating cells). Similarly, neutral comet assay showed dose-dependent elevation of the DNA migration induced in both non-proliferating and proliferating lymphocytes treated with EPX when compared with negative controls. Our results suggest that epoxiconazole fungicide is capable of causing damage to the genetic material of the bovine cells.

Keywords: epoxiconazole, genotoxicity, cattle, comet assay.

INTRODUCTION

Pesticides are a significant source of environmental pollution due to their wide-ranging application in agriculture and forestry. Exposure to these pollutants can have both acute and chronic effects on target and non-target

organisms (Berenzen et al. 2005). Long-term exposure and chronic poisoning with pesticides can trigger genotoxic and epigenetic processes through various pathways, interactions and doses resulting from the intensive use of pesticides and can cumulatively lead to genetic change in humans, covertly and without clinical evidence (Bull et al. 2006). As later indicated by Kaur and Kaur (2018), occupational exposure to pesticides in agricultural workers has been associated with an increased incidence of various diseases such as cancer, Parkinson's disease, Alzheimer's disease, reproductive disorders, and birth defects.

Conazoles are a class of azole-based fungicides which are widely used as pesticides in the cultivation of crops despite their suspected endocrine disrupting properties (Roelofs et al. 2014) but also as human and veterinary pharmaceuticals for the treatment of oropharyngeal, vaginal as well as systemic candida and mycosis infections (Kjaerstad et al. 2010). These fungicides act by inhibiting a specific cytochrome P450 (CYP) enzyme, CYP51 (lanosterol 14α-demethylase), which mediates a critical step in the biosynthesis of ergosterol, a steroid required for the synthesis of the fungal cell wall (Zarn et al. 2003). For this reason they are called demethylation inhibitor (DMI) or ergosterol-biosynthesis-inhibiting (EBI) fungicides. Besides their effects on fungal CYP51, triazole-based conazoles have the potential to interact with the mammalian cytochrome P450 (CYP) system, e.g. via inhibition of aromatase (CYP19) which can lead to numerous toxicological effects (Chambers et al. 2014). As reported by Roelofs et al. (2013) conazoles also cause catalytic inhibition of the CYP17 enzyme, responsible for the conversion of pregnenolone and progesterone to androgen precursors. Exposure to these compounds from multiple environmental matrices can cause many negative effects including carcinogenicity hepatotoxicity, reproductive and developmental toxicities (Goetz and Dix 2009; Hester et al. 2012; Heise et al. 2015; Mu et al. 2016; Heise et al. 2018). In spite of the large production and extensive usage of many conazoles, accurate data on human exposure levels are scarce. Besides occupational and pharmaceutical exposure, individuals can also be exposed to conazoles through environmental, food, resident or bystander exposure. This is confirmed by the increasing concentrations of conazole pesticides found in surface and waste waters (Kahle et al. 2008).

Epoxiconazole (EPX) belongs in the triazole class of pesticides and is used worldwide as a fungicide for plant protection. It is known to combat various target fungal diseases in cereals, rice, sugar beets, bananas, coffee, and soybeans (Passeport et al. 2011). This DMI fungicide was effectively used for the control of Fusarium head blight of wheat in China (Chen et al. 2012). In Europe and Australia, the epoxiconazole is part of several commercially successful one-compound fungicide formulations (Epic, Opus) or two-compound formulations composed from combinations of epoxiconazole with different pesticide (Splice, Swing Gold, Tango Super, Venture etc.). Glyphosate, DDTs and the broad-spectrum fungicides boscalid, epoxiconazole and tebuconazole were the most frequently found in agricultural soil samples of 11 member states of the European Union (EU) (Silva et al. 2019). These findings confirmed the previous study of Hvězdová et al. (2018), where conazoles showed the second most frequent occurrence among currently used pesticides (CUPs) in Central European arable soils. In the Czech Republic, Vašíčková et al. (2019) determined that epoxiconazole was one of the main contributors to the overall pesticide mixture toxicity: the measured levels and its frequent presence in soils represented a risk for the agroecosystems. This contribution might be a result of low biodegradability and photochemical stability of the EPX molecule that makes it very persistent in soil and aquatic sediment (Passeport et al. 2011) and allows entering multiple environmental media through spray drift or surface runoff (Potter et al. 2014).

Bovine farm animals are exposed to chemical agents through grazing, so they are the first in which adverse effects of pesticides might occur (Drážovská et al. 2016). For this reason, in this study, we would like to present new data from an experiment where DNA damage was investigated after exposure of bovine peripheral lymphocytes to epoxiconazole. Both alkaline and neutral comet assays were used as the methods of choice for detection of single-strand and double-strand DNA breaks.

MATERIALS AND METHODS

Blood samples were collected by means of jugular venipuncture from two healthy bulls (Slovak indigenous cattle, 6 month old). The animals were kept in healthy conditions, not treated with any drugs and fed with clean feed. The study was conducted in accordance with national and institutional guidelines for the protection of human subjects and animal welfare. Lymphocytes isolated from whole blood were used for the comet assays.

Epoxiconazole (CAS registry number 133855-98-8, 99% purity, Sigma, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, USA) and introduced into culture flasks at concentrations of 2.5, 5, 10, 25, 50 and 100 μg/mL. The fungicide doses were chosen according to study of Šiviková et al. (2018), where the fungicide cytotoxicity level was identified at a

concentration of more than 100 µg/mL. The final DMSO concentration was 0.1% in both the treated and untreated (negative control) cells. Hydrogen peroxide (H_2O_2, Mikrochem, SR, 250 µM) was used as a positive control agent.

Cell cultivation and treatment

For comet assay, lymphocytes were immediately isolated from bovine whole blood using the Histopaque®-1077 (Sigma-Aldrich, St. Louis, MO, USA) separation medium. Isolated lymphocytes were treated with epoxiconazole for 2 h in two different ways: immediately after isolation (non-proliferating lymphocytes) and for the last 2 h of 48 h cultivation (i.e. pre-cultivation of lymphocytes before 2 h treatment to obtain proliferating lymphocytes). Medium for non-proliferating lymphocytes consisted from 4 ml RPMI 1640 medium supplemented with L-glutamine and 15 µM HEPES, 1 ml bovine foetal serum (BOFES) and 40 µl antibiotic/antimycotic mixture (100 U/mL penicillin, 0.1 mg/mL streptomycin and 0.25 µg/mL amphotericin) (Sigma-Aldrich, St. Louis, MO, USA). Immediately after isolation lymphocytes were added to the medium and exposed to the test fungicide for two hours (2 h) (i.e. concurrently with their addition to the medium) according the procedure of Calderón-Segura et al. (2012).

In the experiment with proliferating lymphocytes phytohaemagglutinin (PHA-L, 20 µg/mL, PAN Biotech, Germany) was added to the above-described culture medium. The isolated lymphocytes were subsequently incubated at 37°C for 48 h and exposed to epoxiconazole for the last 2 h of cultivation.

The cells of positive controls were treated with H_2O_2 (250 mM) for 5 minutes (Horváthová et al. 2006).

Cytotoxicity

After exposure completed, the cells were washed twice with phosphate-buffered saline (Dulbecco A, pH 7.4) and resuspended to a final volume 1mL with PBS. Cytotoxic effects on the bovine peripheral lymphocytes were evaluated using the trypan blue dye exclusion staining (0.4% trypan blue), where the number of viable (shiny) and dead (blue) cells were scored (viability test).

Alkaline comet assay

The alkaline comet assay procedure was the same for both non-proliferating and proliferating lymphocytes. Each concentration tested was represented on special microscope comet slides (CometSlides™ 2-Well, TREVIGEN**,** Gaithersburg, Maryland, US) treated to promote agarose adherence, in this case ready-to-use low melting point agarose (LMPA). The cells were mixed with 0.75% LMPA in PBS. The cell suspension was pipetted onto the agarose layer, fitted with a cover slip and left to set at 4°C. After removal of the cover slips, the microscope slides were immersed in cold lysing solution (2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris, plus 1% Triton X-100) for 1 h at 4°C. The slides were then transferred to a horizontal gel electrophoresis tank with electrophoresis solution (0.3 M NaOH, 1 mM Na_2EDTA, pH>13) for 40 min unwinding at 4 °C, and then electrophoresis was conducted at 25V and 300mA for 30 min. The slides were neutralized two times for 10 min with 0.4 M Tris-HCl (pH=7.4), stained with ethidium bromide (5 µg/mL) on both sides, and fitted with cover slips. All of these steps were carried out in the dark and cold (4°C) to prevent the occurrence of additional DNA damage (Collins 2002).

Neutral comet assay

The slides were lysed in cold lysing solution (2.5 M NaCl, 0.1M disodium ethylene diaminetetraacetic acid (EDTA disodium salt), 10 mM Tris-HCl, pH=9.5, 1% N-lauroylsarcosine sodium salt, 1% TritonX-100) for 1h at 4°C. Then the slides were moved to an electrophoretic tank with TBE buffer in which the "unwinding" was performed for 1 hour, followed by electrophoresis (20V) for 40 min. After electrophoresis, the slides were neutralized in blossom with neutralizing solution (0.4 M Tris, pH=7.4) for 2 x 10 min. After drying, the glasses were stained with ethidium bromide (5 µg/mL) (Gyori et al. 2014).

DNA damage evaluation

Comets were analysed with a Nikon ECLIPSE N*i*-U fluorescence microscope, equipped with a Texas Red single band pass filter. A total of 100 nucleoids per slide (three slides for each concentration - 300 nucleoids) were scored visually and five classes of damage were recorded, from 0 (undamaged) to 4 (maximally damaged) according to DNA fluorescence intensity in proportion comparing the comet tail and head. The scores 0-4 were attributed according to visual analysis of nucleoids. The overall score for each slide was therefore between 0-400 (Collins 2002). The percentage of damaged cells and the extent of % DNA damage in the comet tail were calculated.

Statistical analysis

Statistical analysis was performed using simple analysis of variance (ANOVA, Student's t test), which was used to evaluate % DNA breaks comparing treated and untreated groups (controls).

RESULTS

The results of our analysis of DNA damage using both alkaline and neutral comet assays in non-proliferating (2 h exposure to fungicide) and proliferating (48h cultivation and exposure to fungicide for the last 2h) lymphocytes from bovine peripheral blood after exposure to epoxiconazole at concentrations of 2.5; 5; 10; 25; 50 and 100 μg/mL, are summarized in Fig. 1*a, b* and Fig. 2*a, b*. The percentage viability of non-proliferating and proliferating lymphocytes from bovine peripheral blood following exposure to epoxiconazole is shown in Fig. 3*a, b* (alkaline comet assay) and Fig. 4*a, b* (neutral comet assay).

Regarding the results of alkaline comet assay after 2h exposure of non-proliferating lymphocytes to epoxiconazole, increases in DNA damage with statistical significance were found starting from concentration 5 μg/mL in donor 1 (5 μg/mL * $p<0.05$; 10, 25, 50 and 100 μg/mL ** $p<0.01$; ANOVA and Student's t test; Fig. 1*a*) as well as donor 2 (5 μg/mL * $p<0.05$; 10, 25, 50 and 100 μg/mL ** $p<0.01$; ANOVA and Student's t test; Fig. 1*a*).

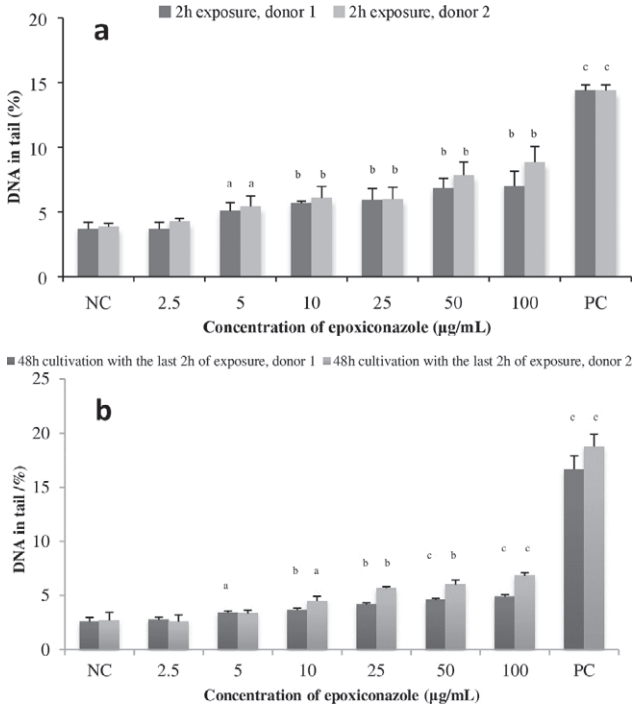

Figure 1. Percentages of DNA in tail estimated by means of alkaline comet assay in bovine peripheral blood lymphocytes (non-proliferating) treated with epoxiconazole for 2 h (a) and in bovine peripheral blood lymphocytes (proliferating 48 h) treated with epoxiconazole for the last 2 h (b). NC (negative control): DMSO; PC (positive control): H_2O_2 (250μM); a: $p<0.05$; b: $p<0.01$; c: $p<0.001$; mean ± SD.

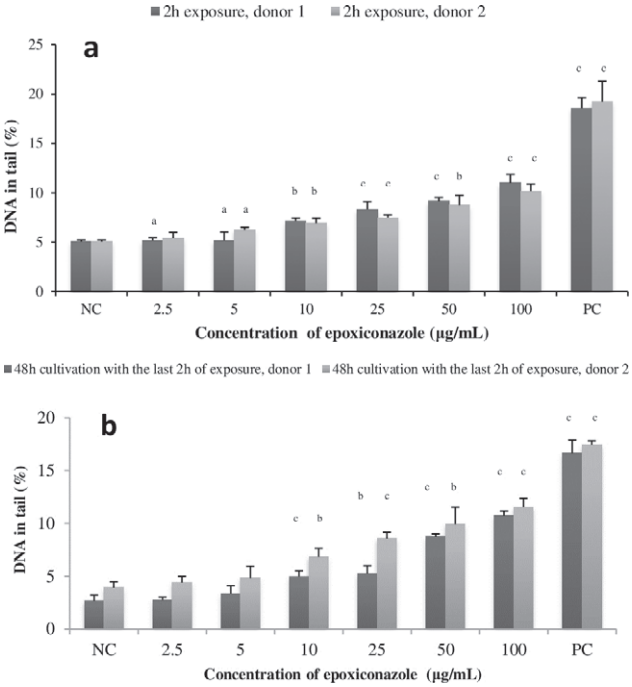

Figure 2. Percentages of DNA in tail estimated by means of neutral comet assay in bovine peripheral blood lymphocytes (non-proliferating) treated with epoxiconazole for 2 h (a) and in bovine peripheral blood lymphocytes (proliferating 48 h) treated with epoxiconazole for the last 2 h. NC (negative control): DMSO; PC (positive control): H_2O_2 (250μM); a: $p<0.05$; b: $p<0.01$; c: $p<0.001$; mean ± SD.

After 48h cultivation and exposure to epoxiconazole for the last 2 h, DNA damage was observed in proliferating lymphocytes with statistical significance in donor 1 (5 μg/mL * $p<0.05$; 10, 25 μg/mL ** $p<0.01$; 50 and 100 μg/mL *** $p<0.001$; ANOVA and Student's t test; Fig. 1*b*) as well as donor 2 (10 μg/mL * $p<0.05$; 25, 50 μg/mL ** $p<0.01$; 100 μg/mL *** $p<0.001$; ANOVA and Student's t test; Fig. 1*b*).

The viability of non-proliferating lymphocytes was greater than 95% in both donors (Fig. 3*a*), and for proliferating lymphocytes it was greater than 94.7% in both donors, too (Fig. 3*b*).

Statistically significant increases in DNA damage with double-stranded breaks in proliferating and non-proliferating lymphocytes were detected using neutral comet assay after exposure to epoxiconazole (Fig. 2*a, b*) at the same concentrations as for alkaline comet assay. Lymphocyte viability is shown in Fig. 4 *a, b*.

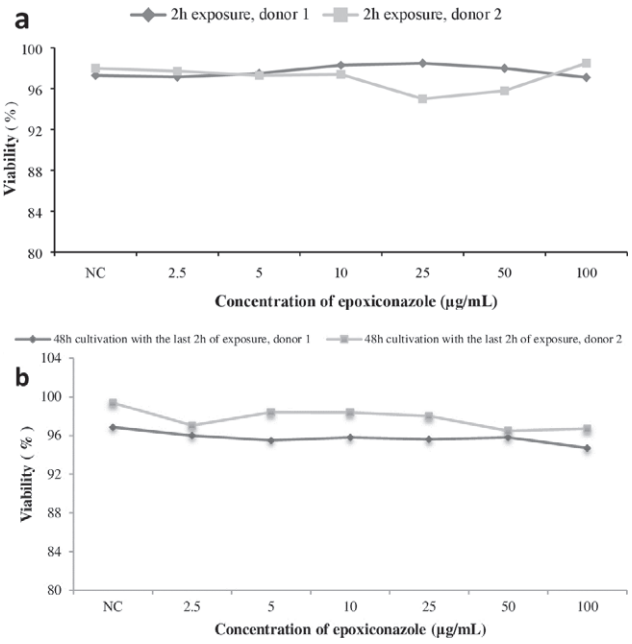

Figure 3. Viability of bovine peripheral blood lymphocytes used in alkaline comet assay. Cells were treated with fungicide epoxiconazole for 2h (a) and for the last 2h of 48h cultivation (b). NC (negative control): DMSO.

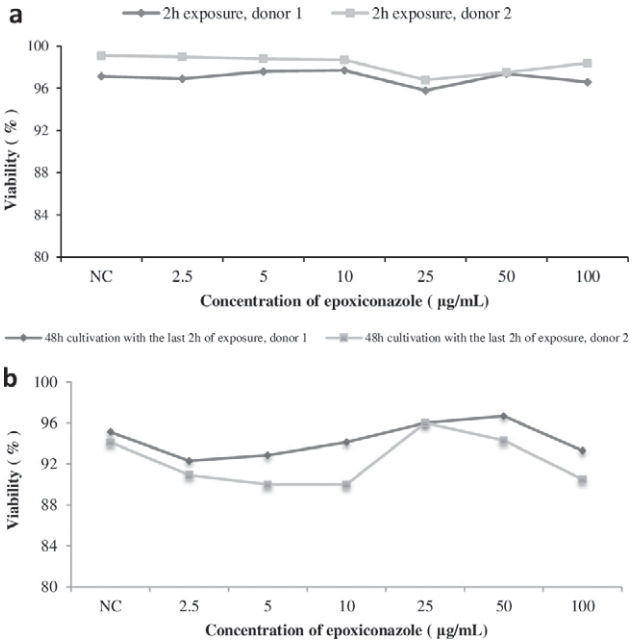

Figure 4. Viability of bovine peripheral blood lymphocytes used in neutral comet assay. Cells were treated with fungicide epoxiconazole for 2h (a) and for the last 2h of 48h cultivation (b). NC (negative control): DMSO.

DNA damage results detected using neutral comet analysis after exposure of non-proliferating lymphocytes to epoxiconazole indicate statistically significant DNA damage in donor 1 from the lowest concentration (2.5 and 5 µg/mL *p<0.05; 10 µg/mL ** p<0.01; 25, 50 and 100 µg/mL *** p<0.001; ANOVA and Student's t test; Fig. 2*a*) and in donor 2 from concentration 5 µg/mL (5 µg/mL * p <0.05, 10, 50 µg/mL ** p <0.01; 25 and 100 µg/mL *** p<0.001; ANOVA and Student's t test; Fig. 2*a*).

Proliferating lymphocytes showed statistical significance in donor 1 from starting from concentration 10 µg/mL (25 µg/mL ** p<0.01; 10, 50 and 100 µg/mL *** p<0.001; ANOVA and Student's t test; Fig. 2*b*) and in donor 2 starting from concentration 10 µg/mL (10, 50 µg/mL ** p<0.01; 25 and 100 µg/mL *** p<0.001; ANOVA and Student's t test; Fig. 2*b*).

Lymphocyte viability found in both donors after epoxiconazole exposure was higher than 95.8% (Fig. 4*a*) in non-proliferating lymphocytes and higher than 90% in proliferating ones (Fig. 4*b*).

DISCUSSION

Comet assay (single-cell gel electrophoresis) is one of the most popular methods employed for the evaluation of DNA damage and repair in eukaryotic cells (Singh 2016; Lu et al. 2017; Moller 2018) This method is used to study processes dealing with DNA damage in various fields, such as environmental toxicology, biological process monitoring, radiation biology, nutritional studies and cancer studies (Olive 2009; Wasson et al. 2008). This test has a wide spread in genotoxicity testing mainly due to advantages such as simplicity of the test, low cost and high sensitivity (Hartmann et al. 2003; Tice et al. 2000). The comet test is a universal and sensitive method measuring single-stranded and / or double-stranded DNA breaks as well as photodimers (Collins et al. 2008). There are two basic variants for determining DNA damage using comet analysis under alkaline or neutral conditions (Östling 1984; Singh 1988). Visual classification of nucleoids and calculation of percentage DNA at the tail is commonly presented up today (Collins et al. 2002; García et al. 2004; Bruschweiler et al. 2016; Hamdi et al. 2018) as an alternative to image analysis.

In the present study, the possible genotoxic and cytotoxic effects of epoxiconazole fungicide were assessed in bovine lymphocytes using alkaline and neutral variants of the comet assay. Treatment was performed on non-proliferating and proliferating lymphocytes to evaluate whether the status of cells has an impact on the DNA damage level. Therefore, we evalu-

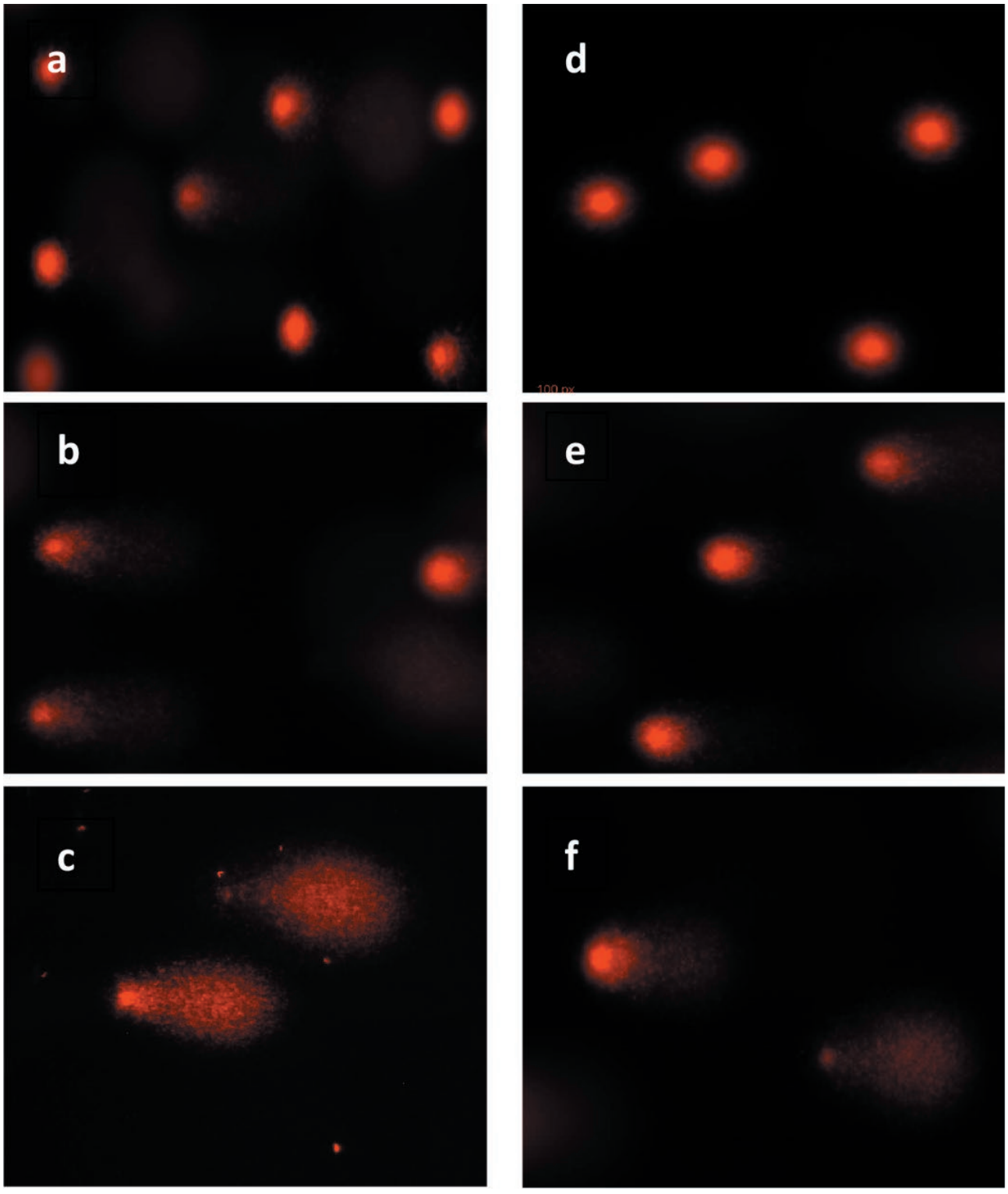

Figure 5. DNAdamage was investigated after exposure of bovine peripheral lymphocytes to epoxiconazole. The cells were treated with the fungicide for 2 h (non-proliferating lymphocytes) and for the last 2 h of the 48-hour culture (proliferating lymphocytes). Positive control was H_2O_2 (5 min). The results of the alkaline comet assay are shown in the first column (picture a, b, c) and the neutral comet assay in the second column (d, e, f). Negative control: a, d. Selected concentration 50 µg/mL: b, e. Positive control: c, f.

ated two different experiments. The first one was with non-dividing (non-proliferating) lymphocytes exposed to EPX immediately after isolation for 2 hours, as indicated by Calderón-Segura et al. (2012). The second with lymphocytes stimulated to divide by phytohaemagglutinin (PHA) during 48 hours taking account the results of Bausinger and Speit (2014) who revealed that DNA synthesis starts in T lymphocytes (similarly like in peripheral blood mononuclear cells, PBMC) around 24 h after stimulation with PHA. Therefore we chose 48-hour (24 h plus 24 h) cultivation allowing lymphocyte proliferation in the medium at least during one cell cycle. We treated the cultured lymphocytes with EPX the last 2 h for the examination of the epoxiconazole ability to induce DNA damage in proliferating cells. Using alkaline comet assay we showed that epoxiconazole induced statistically significant DNA damage in both non-dividing (without PHA) and dividing (with PHA stimulation) lymphocytes of cattle. EPX induced DNA migration in PHA-stimulated cultured lymphocytes in the same range of concentrations like in non-stimulated ones but to a different extent; less DNA migration was observed in PHA-stimulated cells. It is likely that these results correspond with different capability of proliferating cells to repair DNA damage. On the contrast to alkaline comet assay, neutral comet assay showed that DNA breaks were induced in different ranges of concentrations in proliferating (dividing) lymphocytes (from 10 μg/mL) when compared with non-proliferating cells (from 2.5 μg/mL) (Fig. 2*a, b*). One of explanation might be that the lower concentration did not induce DNA damage or that neutral comet assay detects mostly double-strand breaks (Lu et al. 2017), which were probably more effectively repaired in proliferating lymphocytes than in non-proliferating ones.

The results of the comet assay can be affected by the exposure time which is one of a crucial factor. Long incubation periods may not be appropriate for the comet assay because DNA lesions may be repaired during the time that mutagens are inactivated, leading to false negative results (Sekihashi et al. 2003). According to Tice et al. (2000), an appropriate exposure time for chemical *in vitro* genotoxicity assessment should be around 3 to 6 hours; other papers refer 1 h, 2 h, 4 h or 24 h exposure times (Lebaily et al. 1997; Calderón-Segura et al. 2012; Želježić et al. 2016).

It is known that cattle can accumulate foreign substances not only in the liver but also in the muscle (García-Repetto et al. 1997), milk (Pokorná et al. 1996) and fat (Ferré et al. 2018) thereby increasing the genetic risk to humans through the food chain. Guitart et al. (2010) reported that as a result of the application of fungicides in agricultural production, livestock poisoning may occur, the clinical manifestations of which are only rarely addressed. Exposure of livestock to genotoxic substances may also induce mutations, lead to metabolic disorders, immunosuppression and decreased fertility. Cattle are exposed to chemicals during grazing, so adverse effects may occur primarily in them. Besides, some of the chemical agents have a long-term cumulative effect and can contribute to cancer through chronic exposure.

Genotoxicity assessment is an essential component of the safety analysis of all types of substances, ranging from pharmaceuticals, industrial chemicals, pesticides, biocides, food additives, cosmetics ingredients, to veterinary drugs, relevant in the context of international legislation aiming at the protection of human and animal health (ECVAM 2013). As reported by Bolognesi and Morasso (2000) pesticides have been considered potential chemical mutagens. The genotoxicity of pesticides is generally considered to be the most serious of the possible side effects of their usage. The formation of highly reactive substances during oxidation processes, coupled with the ability to interact with DNA, leads to a series of measurable changes, for example point mutations, chromosomal rearrangements, DNA adducts, DNA strand fragments and increased number of micronuclei (Medina et al. 2007). There are several studies testing the genotoxicity of pesticides and mycotoxins on bovine lymphocytes (Lioi et al. 2004; Holečková et al. 2013; Schwarzbacherová et al. 2017; Ferré et al. 2020). Šiviková et al. (2018) tested epoxiconazole *in vitro* in cultured bovine peripheral lymphocytes using chromosome aberrations, sister chromatid exchanges and micronucleus test. She found that epoxiconazole was not related to genotoxic and / or clastogenic / aneugenic effects, but had the ability to significantly affect cell-cycle kinetics and induce apoptosis.

Our results show that epoxiconazole can induce significant levels of DNA damage in bovine lymphocytes, as revealed in both alkaline and neutral variants of the comet assay. On the other hand, no statistically significant DNA damage was detected by Drážovská et al. (2016), who investigated DNA damage using alkaline comet assay after 2 h exposure of bovine lymphocytes to Tango® Super fungicide (epoxiconazole/fenpropimorph). Potential genotoxic/cytotoxic effects of the epoxiconazole/fenpropimorph-based fungicide were also investigated by cytogenetic assays: chromosomal aberrations, sister chromatid exchanges, micronuclei and fluorescence *in situ* hybridization. The final results indicated that the tested fungicide was capable of evoking cytotoxic effect / cell-cycle delay in peripheral cattle lymphocytes. On the contrary Schwarzbacherová et al. (2017)

reported stimulation od DNA-double strand breaks after 4 h exposure to epoxiconazole / fenpropimorph-based fungicide (Tango Super) using neutral comet assay. When compared with pure EPX, the results of both studies mentioned above were probably affected by the presence of fenpropimorph and inert ingredients in the tested pesticide formulation, as well as by different exposure times and variants of comet assay.

Similarly to our observations, significantly increased percentages of comets and tail lengths were obtained after epoxiconazole treatment in the human colon carcinoma cell line (HCT116) (Hamdi et al. 2018). Epoxiconazole was able to induce a range of cell damage in HCT116 cells by generating ROS, which in turn induces mitochondrial DNA dysfunction and fragmentation leading to cell death, as confirmed by the attenuated death of cells treated with the antioxidant N-acetylcysteine (NAC) prior to treatment with epoxiconazole. In the later study with F98 glioma cells the same author (Hamdi et al. 2019) showed that EPX induced cytotoxic effects, cell cycle arrest, cytoskeleton disruption, DNA damage and apoptosis via caspases dependent signalling. In addition Akram et al. (2019) confirmed that epoxiconazole was the potent inhibitor of 11-bhydroxylase (CYP11B1) and aldosterone synthase (CYP11B2) in hamster and human adrenal H295R cells; these enzymes catalyse the formation of cortisol and aldosterone in the adrenal cortex therefore in this study epoxiconazole seems to be an endocrine disruptor. Similarly, Taxvig (2007) concluded that disruption of a crucial enzyme such as CYP17, which is involved in steroid synthesis hormone, is one of the main endocrine-disrupting mechanisms of azole fungicides like tebuconazole and epoxiconazole.

In our experiment, bovine lymphocytes were tested under *in vitro* conditions to obtain significant results, preceded by the testing of several methods and procedures to create optimal experimental conditions. Treatment of cells with epoxiconazole was followed by determination of cell viability for each test concentration using the trypan blue exclusion method, where the percentage of viability represents the number of viable cells compared to the total number of cells surviving after treatment. The cell viability was greater than 90% at all concentrations tested. Tice et al. (2000) recommended that *in vitro* treatment with chemicals should not reduce cell viability by more than 30%, and extended this similarly to *in vivo* experiments. Other researcher maintain that the allowed cell viability after exposure should be at least 70-75% (Želježić et al. 2018), or above 85 % (Evans et al. 2016), and some also report up to 95% (Lebailly et al. 2015) upon performing comet analysis.

In general, the question of determining the sensitivity of both alkaline and neutral comet analysis is frequently discussed, so the comparison of sensitivity of individual methods is interesting from the practical point of view (Afanasieva and Sivolob 2018; Azqueta and Collins 2013; Peycheva et al. 2009; Collins et al. 2008). The alkaline variant demonstrates increased sensitivity in the investigation of agents causing DNA strand breaks or inducing alkaline labile lesions of DNA. Currently, the first choice is to detect low levels of DNA damage, either in lymphocyte samples or in genotoxicity testing *in vitro* and *in vivo*. According to other authors, the neutral variant is more sensitive than the alkaline one. For instance, Afanasieva et al. (2009) indicate that the neutral variant is the more sensitive method for assessing small numbers of DNA breaks.

CONCLUSION

Our data acquired using alkaline and neutral comet assays suggest that epoxiconazole induces significant DNA damage in both non-proliferating and proliferating bovine lymphocytes.

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New genome size estimates for band-winged and slant-faced grasshoppers (Orthoptera: Acrididae: Oedipodinae, Gomphocerinae) reveal the so far largest measured insect genome

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Abstract. Grasshoppers, specifically those of the family Acrididae are known to have the largest genomes of all insects. However, less than 100 species of Orthoptera have their genome size estimated so far. In the present study, we measured the genome size of five acridid species belonging to the two subfamilies Oedipodinae and Gomphocerinae. All of the genomes measured are large and range between 1C = 11.31 pg in the female of *Chorthippus dorsatus* and 1C = 18.48 pg in the female of *Stethophyma grossum*. The latter represents the so far largest measured insect genome. We further provide a summary of genome size estimates available for Orthoptera.

Keywords: C-value, flow cytometry, *Stethopyhma, Oedipoda, Sphingonotus, Chorthippus.*

INTRODUCTION

The genome has become one of the most important targets of interest for biologists. In times of high throughput sequencing, projects like i5k generate data of entire genomes are at a daily base (Robinson et al. 2011; Li et al. 2019). However, we still have little data and a limited understanding of the variance in genome size across organisms. Especially for insects, the most diverse group of organisms on earth, data of only about 1,300 of the expected diversity of several million species are available (Sadílek et al. 2019a; Gregory 2020). Generating new data on genome sizes is important, e.g., for choosing the adequate NGS applications for genomic sequencing (Rodríguez et al. 2017). Yet, genome size can also be a taxonomic feature

and can be used for species determination (Sadílek et al. 2019b). For many applications taxa with specifically large genomes still remain a difficult target, especially if no complete genome sequence is available. Further, in order to understand why some species or species groups have specifically large genomes, whereas others are rather small requires comprehensive data across a large range of taxa.

While the so far largest genome of any organism was estimated in a plant, the monocot *Paris japonica* Franchet with 1C = 152.23 pg (Pellicer et al. 2010), the largest genome sizes in insects have been measured in Orthoptera, specifically Caelifera, with 1C values of 16.93 pg in *Podisma pedestris* (Linnaeus, 1758) (Podismi-nae) and 16.34 pg in *Stauroderus scalaris* (Fischer von Waldheim, 1846) (Gomphocerinae) (Gregory 2020 for a list). However, there is also a lot of variation within Orthoptera with genome sizes as small as 1C = 1.55 pg found in the cricket *Hadenoecus subterraneus* (Scudder, 1861) (Rasch and Rasch 1981). Nevertheless, a clear trend for larger genomes in the short-horned grasshoppers is observed, and specifically in the family Acrididae. In the present study, we were able to locate only 85 published genome size estimates from all Orthoptera (e.g. Gregory 2020).

To better understand the evolution of genome size in Orthoptera, especially the huge genomes of grasshoppers of the Acrididae family, it is obligatory to generate additional information. Hence, we provide new genome size information for members of the Acrididae family, i.e. three species of the subfamily Oedipodinae and two species of the Gomphocerinae. We present, to our knowledge, the so far largest genome size of any insect and summarize the knowledge on genome sizes in Orthoptera.

MATERIAL AND METHODS

Sampling

Eight specimens from five species (Table 1), all of the family Acrididae, were collected for our analyses in September 2019 in Hamburg, Georgswerder (Germany, 53.5097°N 10.0301°E). Specimens were collected by hand and kept alive until further processing. We included two species of the subfamily Gomphocerinae: *Chorthippus dorsatus* (Zetterstedt, 1821) and a species of the *Chorthippus biguttulus* (Linnaeus, 1758) group (a group of three species *C. biguttulus*, *C. brunneus* (Thunberg, 1815), *C. mollis* (Charpentier, 1825), which can only be identified with certainty by male song patterns; our specimen is a female, but according to morphological traits most likely represents *C. biguttulus*), as well as three species of the subfamily Oedipodinae: *Oedipoda caerulescens* (Linnaeus, 1758), *Sphingonotus caerulans* (Linnaeus, 1767), and *Stethophyma grossum* (Linnaeus, 1758) (Table 1, 2).

Reference specimens are deposited in the Zoological Museum Hamburg (ZMH), part of the Center of Natural History (CeNak) under the accession ZMH 2019/21.

Genome size analysis

Nuclear DNA content (2C) was measured by the flow cytometry method (FCM) as in Sadílek et al. (2019a, b) at the Department of botany of Charles University, Prague. The muscle tissue of one hind femur was used for FCM analysis against the plant-internal standard *Pisum sativum* L. "Ctirad" (Fabaceae) with 2C = 9.09 pg (Doležel et al. 1998; Doležel and Greilhuber 2010). Fresh tissue was homogenized and mixed with a leaf of

Table 1. Diploid chromosome number, 2C genome size, sample/standard ratio of both DAPI- and PI-stained samples and GC content of grasshopper species studied. Samples were measured against *P. sativum* standard with 2C = 9.09 pg. F = female, M = male, 2n = male diploid chromosome number, 2C = nuclear DNA content for nuclei with diploid chromosome number, CV = average coefficient of variation for each stain used.

Species	2n	Sex	2C (pg)	Sample/ standard DAPI ratio	Sample/ standard PI ratio	GC content (%)	Sample CV DAPI - PI
Sphingonotus caerulans	22+XX	F	26.63	2.424	2.930	42.14	2.70 - 2.95
Sphingonotus caerulans	22+X0	M	25.12	2.321	2.764	41.87	2.71 - 2.81
Oedipoda caerulescens	22+XX	F	28.39	2.621	3.123	41.88	3.71 - 5.62
Chorthippus dorsatus	16+XX	F	24.14	2.359	2.656	40.82	2.58 - 2.64
Chorthippus biguttulus	16+XX	F	22.62	2.149	2.488	41.35	2.50 - 4.07
Stethophyma grossum	22+XX	F	36.95	3.326	4.065	42.35	3.41 - 4.23
Stethophyma grossum	22+X0	M	34.72	3.172	3.820	42.08	2.19 - 2.84

Table 2. Genome sizes of Orthoptera so far measured. The template of the table was extracted from Gregory (2020); it was complemented with original references and additional studies. References with an * indicate that the original reference could not be accessed and data are extracted only from Gregory (2020). [1]relative genome size - measured with the DAPI - from Morgan-Richards (2005). M= male, F = female, 2C = genome size of the diploid cell, 2n = diploid chromosome number (if sex is not determined, karyotype of the male is presented; in all species the sex determining system is XX/X0, only males of *Podisma pedestris* can be variable with XY/X0), n.a. = not available; FD = Feulgen densitometry, FCM = flow cytometry method; AN = antenna, BR = brain, HE = haemocytes, MS = muscle, OV = ovaries, S = sperm, TS = testes; AC = *Allium cepa* (1C = 16.50 pg), BO = *Bos taurus* (1C = 3.70 pg), BP = *Bellis perennis* (1C = 1.76 pg), DM = *Drosophila melanogaster* (1C = 0.18 pg), DV = *Drosophila virilis* (1C = 0.34 pg), GD = *Gallus domesticus* (1C = 1.25 pg), HS = *Homo sapiens* (1C = 3.50 pg), LM = *Locusta migratoria* (1C = 5.50 pg), MD = *Musca domestica* (1C = 0.90 pg), MM = *Mus musculus* (1C = 3.30 pg), OM = *Oncorhynchus mykiss* (1C = 2.60 pg), PA = *Periplaneta americana* (1C = 3.41 pg), PS = *Pisum sativum* (1C = 4.55 pg), SG = *Schistocerca gregaria* (1C = 8.70 pg).

Family	Subfamily	Species	Sex	1C [pg]	2n	Method	Cell Type	Standard Sp.	References
Suborder: Caelifera									
Acrididae	Acridinae	*Acrida conica*	n.a.	12.55	23	FD	HE	GD, OM	Rasch 1985*
Acrididae	Acridinae	*Acrida conica*	M	10.82	23	FD	TS	GD	Rees et al. 1978
Acrididae	Acridinae	*Caledia captiva*	M	10.9	23	FD	TS	GD	Rees et al. 1978
Acrididae	Acridinae	*Cryptobothrus chrysophorus*	M	9.37	23	FD	TS	GD	Rees et al. 1978
Acrididae	Acridinae	*Schizobothrus flavovittatus*	M	7.5	n.a.	FD	TS	GD	Rees et al. 1978
Acrididae	Catantopinae	*Macrotona australis*	M	8.49	23	FD	TS	GD	Rees et al. 1978
Acrididae	Catantopinae	*Peakesia hospita*	M	10.47	23	FD	TS	GD	Rees et al. 1978
Acrididae	Catantopinae	*Phaulacridium vittatum*	M	10.73	23	FD	TS	GD	Rees et al. 1978
Acrididae	Cyrtacanthacridinae	*Schistocerca cancellata*	M	9.49	23	FD	TS	LM	John and Hewitt 1966
Acrididae	Cyrtacanthacridinae	*Schistocerca gregaria*	n.a.	8.96	23	FD	V	MM	Fox 1970*
Acrididae	Cyrtacanthacridinae	*Schistocerca gregaria*	M	8.71	23	FD	TS	MM	Wilmore and Brown 1975
Acrididae	Cyrtacanthacridinae	*Schistocerca gregaria*	M	8.55	23	FD	TS	LM	John and Hewitt 1966
Acrididae	Cyrtacanthacridinae	*Schistocerca gregaria*	M	8.74	23	FD	S	n.a.	Camacho et al. 2015
Acrididae	Cyrtacanthacridinae	*Schistocerca paranensis*	M	8.63	23	FD	TS	LM	John and Hewitt 1966
Acrididae	Cyrtacanthacridinae	*Valanga irregularis*	M	9.44	23	FD	TS	GD	Rees et al. 1978
Acrididae	Eyprepocnemidinae	*Eyprepocnemis plorans*	M	9.7	23	FD	S	LM	Ruiz-Ruano et al. 2011
Acrididae	Eyprepocnemidinae	*Heteracris adspersus*	M	6.34	23	FD	TS	AC	Gosalvez et al. 1980
Acrididae	Gomphocerinae	*Gomphocerus sibiricus*	M	8.95	17	FD	TS	AC	Gosalvez et al. 1980
Acrididae	Gomphocerinae	*Chorthippus apicalis*	n.a.	12.61	17	FD	TS	GD	Belda et al. 1991*
Acrididae	Gomphocerinae	*Chorthippus biguttulus*	F	11.31	18	FCM	MS	PS	**this study**
Acrididae	Gomphocerinae	*Chorthippus binotatus*	n.a.	10.91	17	FD	TS	GD	Belda et al. 1991
Acrididae	Gomphocerinae	*Chorthippus cf. binotatus*	n.a.	10.35	17	FD	TS	GD	Belda et al. 1991
Acrididae	Gomphocerinae	*Chorthippus brunneus*	M	10.15	17	FD	TS	AC	Gosalvez et al. 1980
Acrididae	Gomphocerinae	*Chorthippus brunneus*	M	9.46	17	FD	TS	MM	Wilmore and Brown 1975
Acrididae	Gomphocerinae	*Chorthippus brunneus*	M	8.55	17	FD	TS	LM	John and Hewitt 1966
Acrididae	Gomphocerinae	*Chorthippus dorsatus*	n.a.	8.34	17	FD	TS	GD	Belda et al. 1991
Acrididae	Gomphocerinae	*Chorthippus dorsatus*	F	12.07	18	FCM	MS	PS	**this study**
Acrididae	Gomphocerinae	*Chorthippus jacobsi*	n.a.	10.84	17	FD	TS	GD	Belda et al. 1991
Acrididae	Gomphocerinae	*Chorthippus jucundus*	n.a.	11.88	17	FD	TS	GD	Belda et al. 1991
Acrididae	Gomphocerinae	*Chorthippus longicornis*	M	8.58	17	FD	TS	AC	Gosalvez et al. 1980
Acrididae	Gomphocerinae	*Chorthippus nevadensis*	n.a.	11.53	17	FD	TS	GD	Belda et al. 1991

Family	Subfamily	Species	Sex	1C [pg]	2n	Method	Cell Type	Standard Sp.	References
Acrididae	Gomphocerinae	*Pseudochorthippus parallelus*	n.a.	14.72	17	FD	TS	GD	Belda et al. 1991
Acrididae	Gomphocerinae	*Pseudochorthippus parallelus*	n.a.	13.83	17	n.a.	n.a.	n.a.	Petitpierre 1996
Acrididae	Gomphocerinae	*Pseudochorthippus parallelus*	M	13.36	17	FD	TS	MM	Wilmore and Brown 1975
Acrididae	Gomphocerinae	*Pseudochorthippus parallelus*	M	12.31	17	FD	TS	LM	John and Hewitt 1966
Acrididae	Gomphocerinae	*Chorthippus scalaris*	n.a.	14.72	17	FD	TS	GD	Belda et al. 1991
Acrididae	Gomphocerinae	*Chorthippus vagans*	M	8.68	17	FD	TS	AC	Gosalvez et al. 1980
Acrididae	Gomphocerinae	*Chorthippus vagans*	n.a.	8.64	17	FD	TS	GD	Belda et al. 1991
Acrididae	Gomphocerinae	*Myrmeleotettix maculatus*	n.a.	13.38	17	n.a.	n.a.	n.a.	Petitpierre 1996
Acrididae	Gomphocerinae	*Myrmeleotettix maculatus*	M	12.66	17	FD	TS	MM	Wilmore and Brown 1975
Acrididae	Gomphocerinae	*Myrmeleotettix maculatus*	M	12.14	17	FD	TS	LM	John and Hewitt 1966
Acrididae	Gomphocerinae	*Omocestus viridulus*	M	13.16	17	FD	TS	LM	John and Hewitt 1966
Acrididae	Gomphocerinae	*Stauroderus scalaris*	n.a.	16.34	17	n.a.	n.a.	n.a.	Petitpierre 1996
Acrididae	Melanoplinae	*Campylacantha olivacea*	F	6.98	n.a.	FCM	BR	GD	Hanrahan and Johnston 2011
Acrididae	Melanoplinae	*Campylacantha olivacea*	M	6.15	n.a.	FCM	BR	GD	Hanrahan and Johnston 2011
Acrididae	Melanoplinae	*Melanoplus differentialis*	M	6.79	23	FCM	BR	PA	Hanrahan and Johnston 2011
Acrididae	Melanoplinae	*Melanoplus differentialis*	n.a.	6.23	23	FD	HE	GD, OM	Rasch unpubl. *
Acrididae	Melanoplinae	*Melanoplus differentialis*	n.a.	3.84	23	FD	OV, TS	BO	Swift and Kleinfeld 1953*
Acrididae	Melanoplinae	*Melanoplus differentialis*	F	7.26	24	FCM	BR	PA	Hanrahan and Johnston 2011
Acrididae	Melanoplinae	*Melanoplus sanguinipes*	n.a.	5.83	23	FD	HE	GD, OM	Rasch unpubl. *
Acrididae	Melanoplinae	*Podisma pedestris*	M	16.93	23/24	FD	S	SG	Westermann et al. 1987
Acrididae	Oedipodinae	*Ailopus thalassinus*	M	6.68	23	FD	TS	GD	Rees et al. 1978
Acrididae	Oedipodinae	*Austroicetes pusilla*	M	6.29	23	FD	TS	GD	Rees et al. 1978
Acrididae	Oedipodinae	*Gastrimargus musicus*	M	9.01	n.a.	FD	TS	GD	Rees et al. 1978
Acrididae	Oedipodinae	*Humbe tenuicornis*	M	8.21	23	FD	TS	LM	John and Hewitt 1966
Acrididae	Oedipodinae	*Chortoicetes terminifera*	M	7.22	23	FD	TS	MM	Wilmore and Brown 1975
Acrididae	Oedipodinae	*Chortoicetes terminifera*	M	5.99	23	FD	TS	GD	Rees et al. 1978
Acrididae	Oedipodinae	*Locusta migratoria*	F	6.44	24	FCM	n.a.	MM	Wang et al. 2014
Acrididae	Oedipodinae	*Locusta migratoria*	n.a.	6.35	23	FD	HE	GD, OM	Rasch 1985
Acrididae	Oedipodinae	*Locusta migratoria*	n.a.	6.27	23	FD	V	MM	Fox 1970
Acrididae	Oedipodinae	*Locusta migratoria*	M	6.09	23	FD	TS	MM	Wilmore and Brown 1975
Acrididae	Oedipodinae	*Locusta migratoria*	M	5.47	23	FD	TS	GD	Rees et al. 1978
Acrididae	Oedipodinae	*Locusta migratoria*	n.a.	5.28	23	FD	S	MD	Bier and Müller 1969*
Acrididae	Oedipodinae	*Oedipoda caerulescens*	F	14.2	24	FCM	MS	PS	**this study**
Acrididae	Oedipodinae	*Sphingonotus caerulans*	M	12.56	23	FCM	MS	PS	**this study**
Acrididae	Oedipodinae	*Sphingonotus caerulans*	F	13.32	24	FCM	MS	PS	**this study**
Acrididae	Oedipodinae	*Stethophyma grossum*	M	17.36	23	FCM	MS	PS	**this study**
Acrididae	Oedipodinae	*Stethophyma grossum*	F	18.48	24	FCM	MS	PS	**this study**
Morabidae	Morabinae	*Warramaba virgo*	n.a.	4	15	FD	BR	GD	White and Webb 1968

Family	Subfamily	Species	Sex	1C [pg]	2n	Method	Cell Type	Standard Sp.	References
Morabidae	Morabinae	*Warramaba virgo*	n.a.	3.75	15	n.a.	n.a.	n.a.	Petitpierre 1996
Suborder: Ensifera									
Anostostomatidae	Deinacridinae	*Hemideina crassidens* [1]	M	5.4	15	FCM	AN	BP	Morgan-Richards 2005
Anostostomatidae	Deinacridinae	*Hemideina crassidens* [1]	F	6.01	16	FCM	AN	BP	Morgan-Richards 2005
Anostostomatidae	Deinacridinae	*Hemideina thoracica* [1]	M	5.95	15	FCM	AN	BP	Morgan-Richards 2005
Anostostomatidae	Deinacridinae	*Hemideina thoracica* [1]	F	6.53	16	FCM	AN	BP	Morgan-Richards 2005
Gryllidae	Gryllinae	*Acheta domesticus*	n.a.	2.38	11	FIA	HE	DM	Koshikawa et al. 2008
Gryllidae	Gryllinae	*Acheta domesticus*	n.a.	2	11	FD	HE	GD, OM	Rasch 1985
Gryllidae	Gryllinae	*Acheta domesticus*	n.a.	2	11	FD	OV, TS	MM, HS	Lima-de-Faria et al. 1973
Gryllidae	Gryllinae	*Acheta domesticus*	n.a.	2	11	FCM	BR	DM	Gregory unpubl.
Gryllidae	Gryllinae	*Acheta domesticus*	n.a.	2	11	FIA	HE	GD	Gregory unpubl.
Gryllidae	Gryllinae	*Gryllus pennsylvanicus*	n.a.	2.68	11	n.a.	n.a.	n.a.	Petitpierre 1996
Gryllidae	Gryllinae	*Gryllus pennsylvanicus*	n.a.	2.06	21	FD	S	MD	Bier and Müller 1969
Gryllidae	Gryllinae	*Gryllus pennsylvanicus*	n.a.	2	21	FD	HE	GD, OM	Rasch 1985
Gryllidae	Oecanthinae	*Oecanthus niveus*	n.a.	1.71	n.a.	FCM	BR	DV	Hanrahan and Johnston 2011
Gryllotalpidae	Gryllotalpinae	*Neoscapteriscus borellii*	n.a.	3.41	n.a.	FCM	BR	GD	Hanrahan and Johnston 2011
Rhaphidophoridae	Ceuthophilinae	*Ceuthophilus stygius*	n.a.	9.55	n.a.	FD	HE	GD, OM	Rasch and Rasch 1981
Rhaphidophoridae	Ceuthophilinae	*Hadenoecus subterraneus*	n.a.	1.55	n.a.	FD	HE	GD, OM	Rasch and Rasch 1981
Tettigoniidae	Conocephalinae	*Conocephalus sp.*	M	2.65	33	FCM	BR	GD	Hanrahan and Johnston 2011
Tettigoniidae	Conocephalinae	*Conocephalus sp.*	F	3.03	34	FCM	BR	GD	Hanrahan and Johnston 2011
Tettigoniidae	Conocephalinae	*Neoconocephalus triops*	M	7.29	n.a.	FCM	BR	GD	Hanrahan and Johnston 2011
Tettigoniidae	Conocephalinae	*Neoconocephalus triops*	F	7.93	n.a.	FCM	BR	GD	Hanrahan and Johnston 2011
Tridactylidae	n.a.	unknown sp.	n.a.	2.63	n.a.	FCM	BR	DV	Hanrahan and Johnston 2011
Trigoniidae	Trigonidiinae	*Laupala cerasina*	n.a.	1.93	n.a.	FCM	BR	GD	Petrov et al. 2000

the standard in 500 µl of 4°C cold Otto buffer I. The suspension of released cells was then filtered through a 42 µm nylon mesh and divided in two parts. One part was stained with 1,000 µl DAPI solution (stock: 25 ml Otto buffer II, 1 ml DAPI (0.1 mg/ml), 25 µl 2-mercaptoethanol (2 µl/ml)); the second part was stained with 1,000 µl propidium iodide (PI) solution (stock: 25 ml Otto buffer II, 1 ml RNase (1 mg/ml), 1 ml PI (1 mg/ml), 25 µl 2-mercaptoethanol) (Doležel et al. 2007).

For DAPI analysis, the Partec CyFlow instrument (Partec GmbH, Münster, Germany) with UV LED chip and for PI analysis the Partec SL instrument with a green solid-state laser (Cobolt Samba, 532 nm, 100 mW) were used. Each sample was stained for several minutes before measurement, and 3,500 to 5,000 particles were recorded in each FCM analysis. FCM data were analysed with the Partec FloMax v. 2.52 software (Partec GmbH, Münster, Germany).

Combined DAPI and PI measurement results of the same sample express the AT/GC ratio of the genome of the species, the GC content (e.g. Šmarda et al. 2008; Sadílek et al. 2019a, b). The GC content of *P. sativum* is 38.50% (e.g. Barrow and Meister 2002; Šmarda et al. 2008) and the GC content of the analysed samples was calculated with the Microsoft Excel macro from Šmarda et al. (2008).

RESULTS

DAPI-stained samples yielded a lower coefficient of variation (CV) than PI-stained samples, on average CV = 2.83% and 3.59% respectively. All the analysed species of Oedipodinae reached higher genome size values than the analysed species of Gomphocerinae. We were able to measure the genome size of both sexes only in two species (*S. caerulans* and *S. grossum*). There, the female/male genome size values clearly reflected the XX/X0 sex determination system differences. Due to this sex determination system it is generally preferred to report genome size in 2C values rather than the commonly used 1C value. However, to allow for better comparability, we here report both values.

All analysed species of Oedipodinae had distinct genome size (Table 1). The male of *S. caerulans* had 2C = 25.12 pg (1C = 12.56 pg); the female had 2C = 26.63 pg (1C = 13.32 pg). The female specimen of *O. caerulescens* exhibited a 2C value of 28.39 pg (1C = 14.20 pg). The largest genome size was recorded in *S. grossum*, where the male reached 2C = 34.72 pg (1C = 17.36 pg) and the female 2C = 36.95 pg (18.48 pg). Both closely related Gomphocerinae species showed very similar genome sizes (Table 1): 2C = 22.62 pg (1C = 11.31 pg) in the *C.* cf. *biguttulus* female and 2C = 24.14 pg (1C = 12.07) in the female of *C. dorsatus*.

The sample/standard ratio of samples stained with PI was always higher than in DAPI-stained samples of the same specimen, ranging from 11% difference in the female of *C. dorsatus* to 18% difference in the female of *S. grossum*. This trend is observable also in the GC content, where *C. dorsatus* had only 40.82% and the female of *S. grossum* had 42.35% (Table 1). However, the GC content differences among all species analysed were minimal.

DISCUSSION

We present new genome size estimates for five species of Acrididae, one of which represents the largest genome of all insects measured so far, the genome of the female of *Stethophyma grossum* with 2C = 36.95 pg (1C = 18.48 pg). We also measured a female of *C. dorsatus* with 2C = 24.14 pg (1C = 12.07 pg). This species was measured before using the Feulgen densitometry method with 1C = 8.34 pg (Belda et al. 1991). However, the more recent method of flow cytometry we used is considered more accurate for genome size estimations (e.g. Doležel and Greilhuber 2010). Furthermore, we collected all previous estimates from Gregory (2020) and added few additional resources to provide some basic visualization of the genome size variation in the different subfamilies of Orthoptera (Fig. 1).

In total, we gathered 92 (our new data included) estimates of genome sizes belonging to 54 species (Table 2, Fig. 1). These data included 68 estimates for Caelifera (43 species) and 17 for Ensifera (11 species). They ranged from 1C = 3.75 pg for *Warramaba virgo* (Key, 1963) (Morabidae) (Petitpierre 1996) to 1C = 18.48 pg for *Stethophyma grossum* (Oedipodinae, present study) in Caelifera and from 1C = 1.55 pg for *Hadenoecus subterraneus* to 1C = 9.55 pg for *Ceuthophilus stygius* (Scudder, 1861) (both cave Rhaphidophoridae) in Ensifera (Rasch and Rasch 1981). Average 1C values in Ensifera and Caelifera are 3.16 pg (± 2.18 pg) and 9.83 pg (± 3.32 pg) respectively. Further analyses at the family and subfamily level are difficult, as most data comes from Acrididae with 66 measurements (78%). The average genome size in Acrididae is 10.01 pg (± 3.19 pg). Within Acrididae, most estimates came from 26 measurements of Gomphocerinae and 17 of Oedipodinae with average genome sizes of 1C = 11.52 pg (± 2.17 pg) and 9.13 pg (± 4.20 pg) respectively (Table 2, Fig. 1).

Generally, the short-horned grasshoppers (Caelifera) appear to have larger genomes compared to the long-

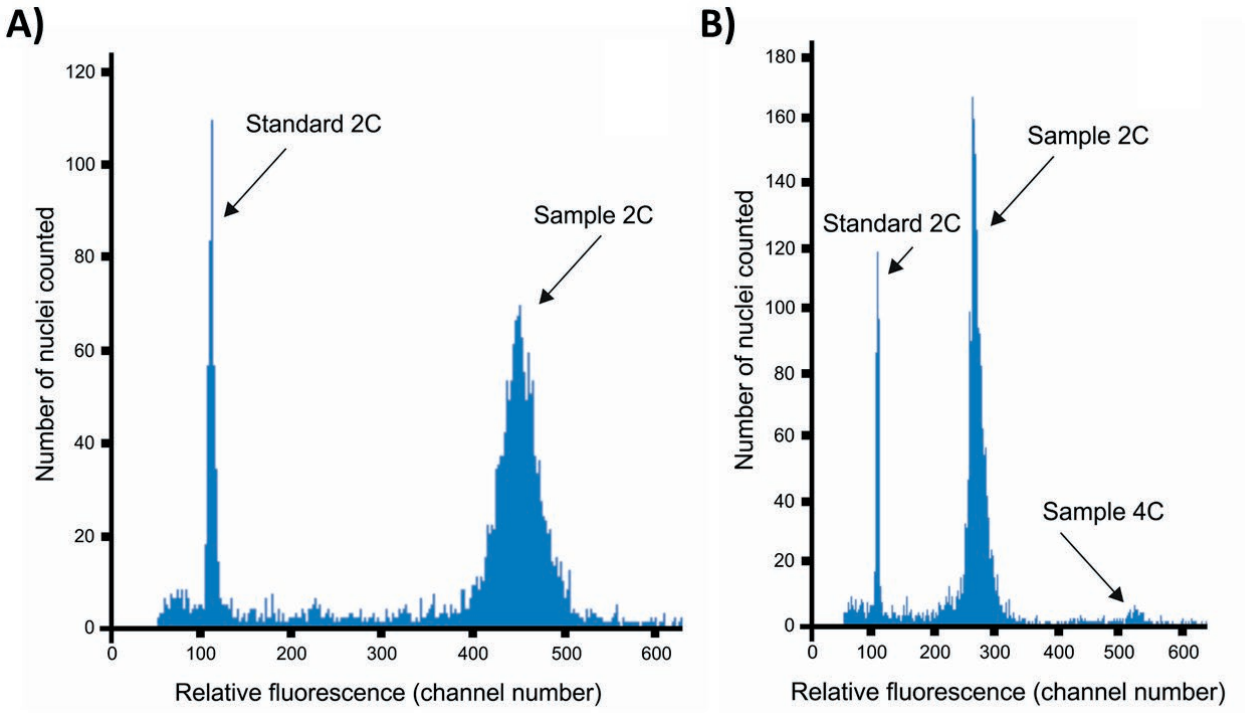

Figure 1. Relative fluorescence histograms for samples stained with PI. 2C peaks represent diploid cells and 4C peaks represent cells in the G2 phase of the cell cycle. with replicated DNA. Standard used: *P. sativum* 2C = 9.09 pg. (**A**) *S. grossum* female with 2C = 36.95 pg. (**B**) *C. biguttulus* female with 2C = 22.62 pg.

horned grasshoppers (Ensifera). However, this is not correlated with the number of chromosomes. Despite their relatively low male number of chromosomes of 2n = 17 (most of other Acrididae have 2n = 23; e.g. Sylvester et al. 2019), Gomphocerinae have some of the largest genome sizes. Their average genome size is 1C = 11.52 pg ranging from 1C = 8.34 pg in *C. dorsatus* (Belda et al. 1991) to 16.34 pg in *Stauroderus scalaris* (Petitpierre 1996; Gregory 2020). Moreover, they show large intraspecific variation in genome size evident from different studies (Table 2), for example: 1C = 12.31 pg to 14.72 pg for *Pseudochorthippus parallelus* (Zetterstedt, 1821) (John and Hewitt 1966; Wilmore and Brown 1975; Belda et al. 1991; Petitpierre 1996) or 1C = 8.55 to 10.15 pg for *C. brunneus* (John and Hewitt 1966; Wilmore and Brown 1975; Gosalvez et al. 1980). All studies of the two species mentioned above share the method of Feulgen densitometry and used testes to measure genome size. Hence it remains unclear whether this variation is natural or the result of methodological differences. However, it is more likely that the large intraspecific differences are a result of a combination of multiple factors: different populations analysed, lack of chromosome observations, various standards used and also different instrumentation could play some role.

The variation in genome size is even higher in Oedipodinae with a minimum of 1C = 5.28 pg for *Locusta migratoria* (Linnaeus, 1758) (Bier and Müller 1969) and a maximum of 1C = 18.48 pg in *Stethophyma grossum*. Hence, *S. grossum* represents the so far largest measured confirmed insect genome. A study by Schielzeth et al. (2014) measured much larger genome sizes for the Gomphocerinae species *C. biguttulus* with 1C up to 236.05 pg. Due to the enormous variation of the estimates in the study and critical methodological issues, Camacho (2016) suggested that these estimates cannot be considered reliable. Hence, we consider our estimate of the *S. grossum* genome size as the current upper size of insect genomes. Since only very few species have been measured so far, it is expected that this is not the upper bound for genome sizes in grasshoppers or for insects in general.

The reasons for the large size of Caelifera genomes remain largely unknown. However, a recent paper by Shah et al. (2020) suggests that repetitive DNA and especially the expansion of satellite DNA may be a main reason for the large genomes in Orthoptera. The most likely causes are genome duplications at the basis of the Acrididae, which would also explain their specifically high rates in nuclear mitochondrial pseudogenes (numts,

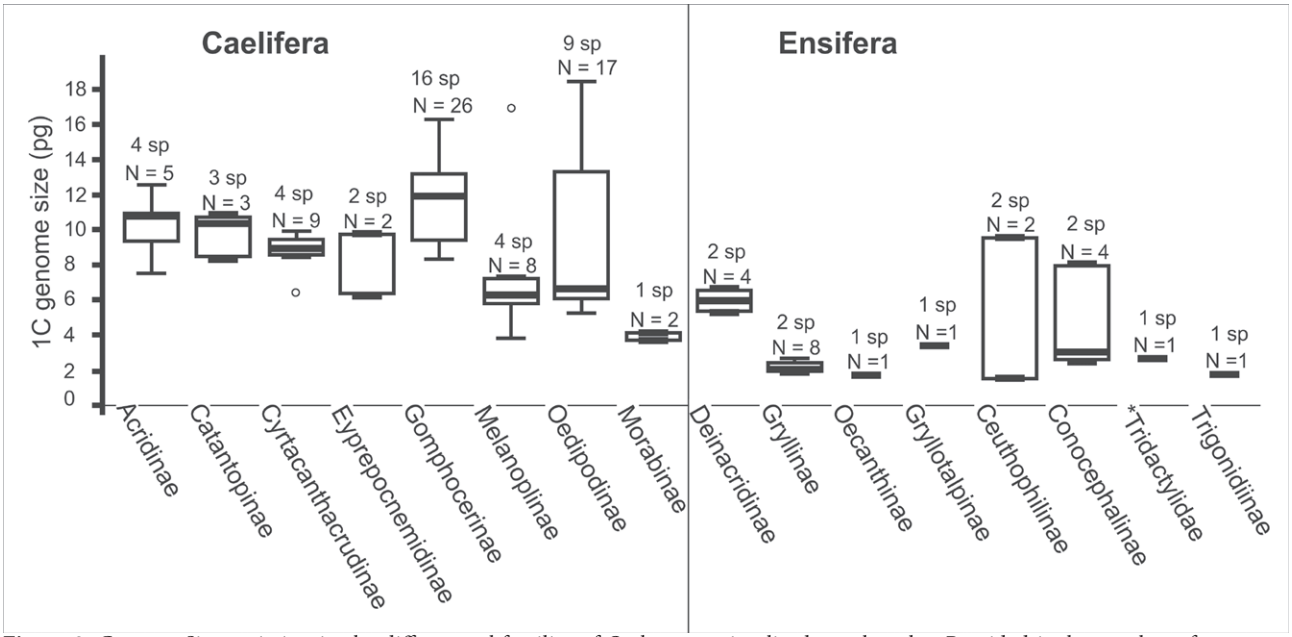

Figure 2. Genome Size variation in the different subfamilies of Orthoptera visualized as a boxplot. Provided is the number of measurements (N) and the number of species (sp) these measurements were derived of (some of the species were measured repeatedly by different authors). Most of the data excerpted from database Gregory (2020) completed with another original data comprehended in Table 2. *unknown species genome size was analysed, determined only on family level.

Bensasson et al. 2000; Song et al. 2008) posing difficulties to species identification using DNA barcoding and to phylogenetic reconstruction (Hawlitschek et al. 2017, Song et al. 2018). It may also explain why only a single incomplete genome is available to date (Wang et al. 2014). Grasshopper genome sizes remain a major obstacle to genomic research, and many further studies will be required to understand genome size variation and evolution in Orthoptera.

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DATA AVAILABILITY STATEMENT

All data generated and used in this article is included as tables and figures.

GEOLOCATION INFORMATION

All sampling for this study was performed 2019 in Hamburg, Georgswerder (Germany, 53.5097°N 10.0301°E).

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Awn microstructural observation revealing multifunction of awn-inhibitor Gene *B1* in near-isogenic lines with different awn length

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Abstract. Awn is one of wheat morphological characteristics and acts as a highly effective organ for photosynthesis in wheat. Variation in awn length is controlled primarily by three major genes, most commonly the dominant awn suppressor *Tipped1* (*B1*). So far, the function of *B1* is not well understood. In this paper, we identified a pair of near-isogenic lines (NILs) containing different awn inhibition gene *B1* alleles and observed microstructures and ultra-microstructure of their awns. The typical awns differences between the NILs represented by the cross-sectional area and chloroplasts number. Long awn line had a larger cross-sectional area, and more cells in various parts of tissues, especially the cells containing more and larger chloroplasts, which could attribute to a strong cytological basis for photosynthesis. The results may suggest that the gene has pleiotropic effects in the control development of awn tissue structure and grain yield.

Keywords: Common wheat, NILs-*B1*, awn length, SEM, anatomy.

INTRODUCTION

Wheat awn is a long and stiff filamentous prolongation of the lemma, which plays an important role in seed dispersal, burial, transpiration and photosynthesis (Grundbacher, 1963; Elbaum et al., 2007). It is one of the morphological characteristics of wheat and other species, such as barley (*Hordeum vulgare*), rye (*Secale cereal* L.), oat (*Avena sativa* L.), rice (*Oryza sativa* L.) and sorghum (*Sorghum bicolor* (L.) Moench). Awn has prickly barbs on its surface, which aid the seed dispersal by attaching to the animal fur (Yuo et al. 2012). In wild wheat, each spikelet has a pair of long awns, which can be guided to fall an appropriate germination site with the balance provided by awn. Moreover, the awns would bend as they dry and straighten in a damp environment, these movement driven by the daily humidity cycle propels the seeds into the ground (Elbaum et al. 2007). Awn is an important

organ of gramineous crop panicle, which ensures wheat adapt to some special environment such as warm growing regions (Motzo & Giunta, 2002). Therefore, it has important biological significance, but its function and genetic research are not well understood.

Genetic analysis has shown that the awnless trait dominated over the awned ones. Three dominant inhibitor alleles, *Hooded* (*Hd*), *Tipped 1* (*B1*), and *Tipped 2* (*B2*), were subject to simple Mendelian inheritance, which has been identified in common wheat (Sourdille et al. 2004; Mackay et al. 2014). *Hd* was located on the short arm of chromosome 4A and resulted in short and broad awns that are curved inward into a hood shape. Previous studies have shown that the effect of the wheat *B1* gene on the length of awn is the strongest, results in very short or absent awns at the base and the middle of the ear (Yoshioka et al. 2017; DeWitt et al. 2020; Huang et al. 2020; Wang et al. 2020). Recent fine mapping located *B1* to a region on the long arm of chromosome 5A containing only two predicted genes, including C2H2zinc finger transcriptional repressor TraesCS5A02G542800 (DeWitt et al. 2020). Another group also reported that *TraesCS5A02G542800* encoding a C2H2 zinc finger protein putatively functions as a transcriptional repressor predominantly responsible for awn inhibition in wheat (Huang et al. 2020; Zhang et al. 2020) identified 4 SNPs in the promoter region of *TraesCS5A02G542800* and proved the *TraesCS5A02G542800* promoter as the control gene of *B1* awn length inhibition site, which was named as *ALI-1* (Wang et al. 2020). All in all, there is a consensus that the *B1* gene is located in the candidate gene or promoter, but its function and regulation mechanism is still uncharacterized.

In the study, line SN051-1(long awn) and line SN051-2(short awn) are NILs with the only difference of awn-length (Du et al. 2010). Genetic analysis indicated that awn from the NILs was controlled by awn-inhibitor gene *B1*. We used optical microscope and Scanning electron microscopy (SEM) to observe the cross-sections, epidermal tissues, and anatomic features of awns and revealed their differences among NILs-*B1*. At the same time, we investigated the photosynthetic rate of the ear and thousand-grain weight of the NILs. The investigation on NILs-*B1* is expected to lay a foundation for the study on the function, genetic mechanism of the awn in common wheat.

MATERIALS AND METHODS

Plant material and growth conditions

A pair of wheat lines with different awn lengths, SN051-1 (long awn, *b1b1*) and SN051-2 (short awn, *B1B1*), were used in the current study. They were developed from the F_8 progeny of Octoploid *Triticale* Jinsong49 and Octoploid *Tritittrigia* Xiaoyan7430, and then were self-crossed for 5 generations (Du et al. 2010). The morphological, genetic and molecular marker analyses showed that they differed in the wheat awn inhibition gene *B1*: line SN051-2 contained the dominant allele *B1*, and SN051-1 possessed the recessive allele *b1*. The hybrid was obtained from the cross of SN051-1 × SN051-2, F_2 population and two back-crosses BC_1F_1 populations including SN051-1/SN051-2//SN051-1) and (SN051-2/SN051-1//SN051-1) BC_1 were developed to study genetic of awn length. All the materials, including SN051-1, SN051-2, their F_1, F_2, and BC_1F_1 hybrid, were grown in two replications as 1 m rows spaced 30 cm apart at Tai'an, Shandong Agricultural University. At heading stage, presence or absence and awn length of the materials were studied. Thousand-grain weight of the lines were investigated at the harvest stage.

SSR Polymorphic Analysis

Total DNA was extracted by the SDS-phenol method (Liu Cheng et al. 2006). The primers used included Xgwm, Xgdm, WMC, BARC, CFA, CFD, STS-MAG, EST-KSUM, EST-CWES, and EST-DUPW in 1690 pairs. The sequence and location of the primers used can be found at http://wheat.pw.usda.gov, and the primers used were synthesized by Shanghai bioengineering co. LTD. The PCR system was 15μl, including 10×Buffer 1.5μl, 25 mmol/L $MgCl_2$ 1.2μl, 2.5 mmol/L dNTP 0.9μl, 25 ng/ 1l primer 3μl, 5U/μL *Taq* enzyme 0.12μl, deionized water 5.28μl, 80 ng/1μl genomic DNA 3μl. Amplification program reference Hao method (Hao et al. 2008), 94 °C modified 4 min, then 15 cycles touch down PCR process sequence, 94 °C modified 45s per cycle, 65 °Crenaturation 50 s diminishing per cycle (1 °C) and 72 °C 55s extension, the final 30 cycles of ordinary PCR process sequence is 94 °C modified 40s, 40s, 50 °C renaturation 72 °C extends 40s, then, it was extended at 72 °C for 5min, and stored at 10 °C after amplification. The amplified products were electrophoresis with 6% nondenatured polyacrylamide gel and stained with silver nitrate.

Microstructure observation of awn and its cross-section

Ten days after the anthesis, awns were collected from spikes of 2 or 3 plants and immediately fixed in 2.5% glutaraldehyde solution in 0.1 M sodium phosphate buffer (pH 7.0) overnight at room temperature, post-fixed with 1% (w/v) osmium tetroxide in phos-

phate buffer at 4 °C, and then embedded in Epon812 (Shell Chemical, Houston, TX, USA) following a standard dehydration procedure. Transverse sections (about 1 cm from the base of the awn, 2.5 μm thick, were cut with an LKB-V microtome, and then stained in 1% (w/v) toluidine blue in 1% (w/v) disodium tetraborate, and observed under an optical microscope (Olympus BX-51, Japan) with automatic camera.

Determination of photosynthetic rate in ear

The photosynthetic rate of flag leaf was determined by LI-6400 portable photosynthesis systems, the panicle photosynthetic rate was measured by GXH-305 infrared CO_2 gas analyzer and a special assimilation chamber on May 25. Each treatment was repeated three times.

RESULT

Genetic analysis of awn length phenotype

SN051-1 and SN051-2 showed no significant difference on plant morphology, but only on awn length, F_1 hybrids presented short awn (Figure 1), F_2 individuals separated into two groups (short awn phenotype, long awn phenotype) which fitting a 3:1 segregation ratio, BC_1 individuals separated into two groups (short awn phenotype, long awn phenotype) which generally fitting a 1:1 segregation ratio, which were consistent with Mendelian segregation of a single gene (Table 1). This result indicated a single genetic locus, represented here as *B1*, which was associated with the dominant short awn phenotype that could be used as a phenotypic marker.

Genetic background and difference analysis based on DNA level on the parents

The F_2 population was analyzed with polymorphic SSR primer Xgwm291, and a band of 140 bp was amplified in SN051-1 and most F_2 long-awn plants, and a band of 110 bp was amplified in SN051-2 and most F_2 short-awn plants (Figure 2). The marker has been located at the end of the long arm of 5A chromosome in wheat, and the genetic distance between the marker and the *B1* of awn length suppressor gene at the end of 5A chromosome is 2.0~4.5cm (Paillard et al. 2003; Somers et al. 2004). According to the results of the molecular marker and the characterization between near-isogenic gene line, SN051-1 and SN051-2 are the near-isogenic gene lines of awn-length inhibiting gene *B1*. This is consistent with the study of the *B1* gene by DeWitt (2019) and Wang DZ (2020).

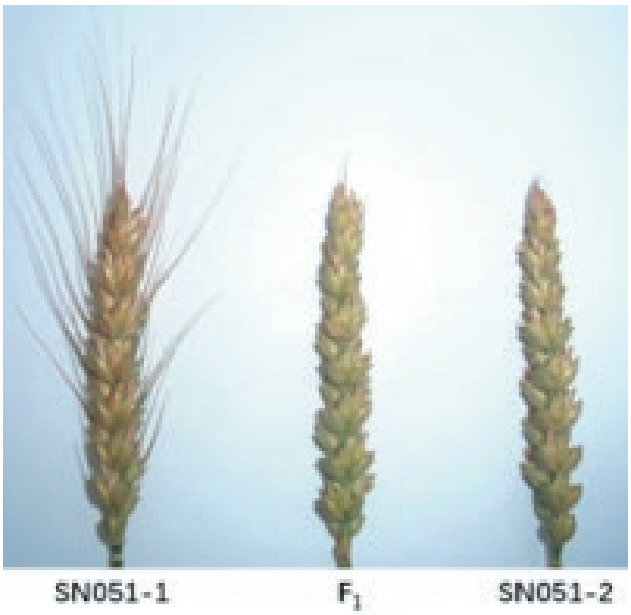

Figure 1. Awn performance of SN051-1, F_1, and SN051-2. SN051-1: long awn phenotype; SN051-2: short awn phenotype; F_1: short awn phenotype.

In a total of 1690 pairs of SSR and 209 sequence-tagged sites (STS), primers were selected for genomic DNA polymorphism analysis of SN051-1 and SN051-2. A total of 64 SSR primer pairs and 7 STS primers were

Table 1. Segregation for long awn or short awn in F_2 populations derived from F_1 of SN051-1 × SN051-2.

Cross combination and generation	Plant number	Observed		Expected		χ^2	Segregation ratio
		As	Ad	As	Ad		
(SN051-1/SN051-2) F_2	536	401	135	402	134	0.00995	3:1
(SN051-2/SN051-1) F_2	456	347	109	342	114	0.292	3:1
(SN051-1/SN051-2//SN051-1) BC_1	142	75	67	71	71	0.45	1:1
(SN051-2/SN051-1//SN051-1) BC_1	128	73	55	64	64	2.531	1:1

Ad: awned; As: awnless.

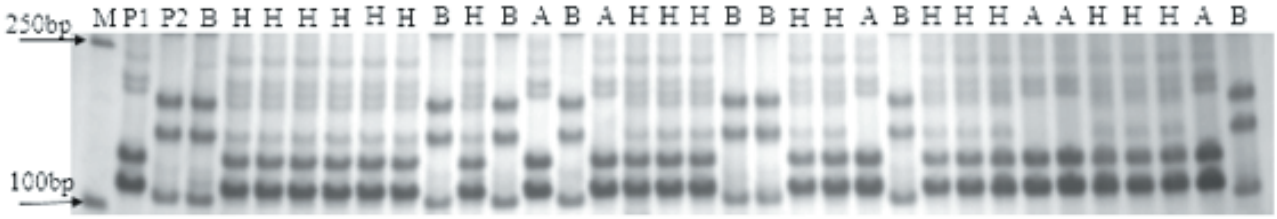

Figure 2. Amplification results with primer Xgwm291 in parents and the F_2 population. M: DL2000 marker; P1: SN051-2; P2: SN051-1; B: F_2 individuals with long awn in; H: F_2 individuals with a short awn.

amplified difference between the two materials, accounting for the proportion of polymorphism primers of 3.79% and 1.38%, respectively, which reflected the similar genetic background and the mine genetic difference between the two lines. Other than SSR and STS markers, we also used Specific-locus amplified fragment sequencing (SLAF-seq) method to verify the single nucleotide polymorphisms (SNP) difference between SN051-1 and SN051-2. The results showed that only 3,679 tags among 216,192 tags produced were accounted for about 1.7% of polymorphic percentage, which also illustrated the mine difference. Therefore the combination of the phenotype characteristics and DNA genetic analysis, SN051-1 and SN051-2, can be considered as near iso-genic lines.

Awn primordial development of the NILs

Besides the awn length trait, we further investigated other awn related traits on the parental pair lines. Awns emerge from the lemma of young spikelets at an early developmental stage. Awn development process in a spikelet (Figure 3) showed SN051-1, and SN051-2 appeared awn primordial without differences on pistil and stamen differentiation stage (Figure 3A). while further develop to anther connective formation stage (Figure 3B), awn primordial of SN051-1 elongated and grew longer, while the awn length of SN501-2 stopped elongating. On the tetrad formation stage, the awn length difference became more obvious, with the characteristics of SN051-1 having long awn and SN051-2 short awn. Scanning electron microscopy (SEM) observation indicated the two NILs significant differences in awn development.

Awn external surface and cross-section features of NILs

Awn external surface contained several stomata and hairs in both SN051-1 and SN051-2, while the number of stomata in a unit area of SN051-2 awns had more stomata and SN051-1 awns had more hairs (Figure 4A). The cross-section of awn contains vascular bundles, parenchyma, and sclerenchyma in NILs (Figure 4B), while SN051-1 awns displayed more areas for each tissue, more complete cell structure and more regular cell arrangement. Moreover, the difference showed by morpho-anatomical structure using the semi-thin section was more obvious (Figure 4C). The above results suggest that SN051-1 has a more obvious xerophytic structure.

Chloroplast in awn cell of NILs

Observation of the ultrastructure of chloroplast in awn cell by transmission electron microscopy (TEM) showed that chloroplast arranged along the cell wall and

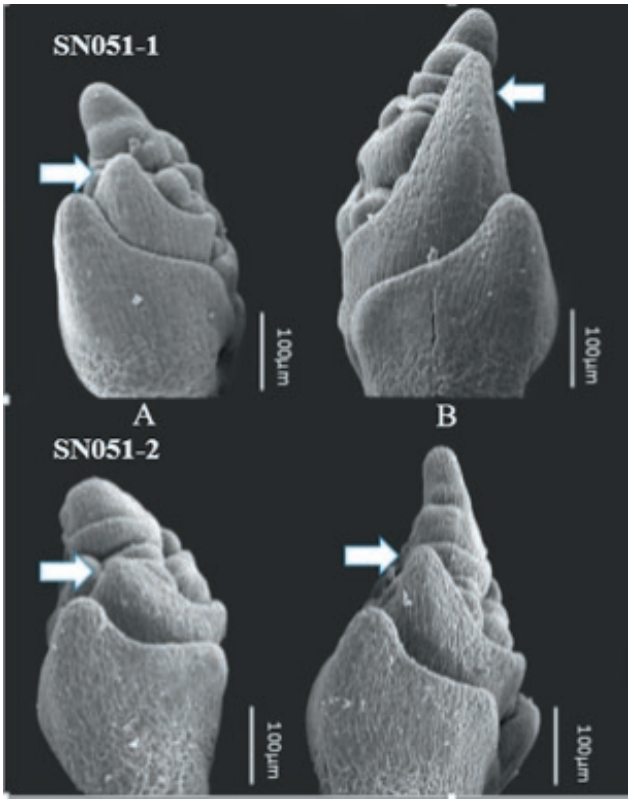

Figure 3. SEM images of SN051-1 and SN051-2 spikelets at different development stages. Arrows point to awn primordia; A pistil and stamen differentiation stage; B anther connective formation stage.

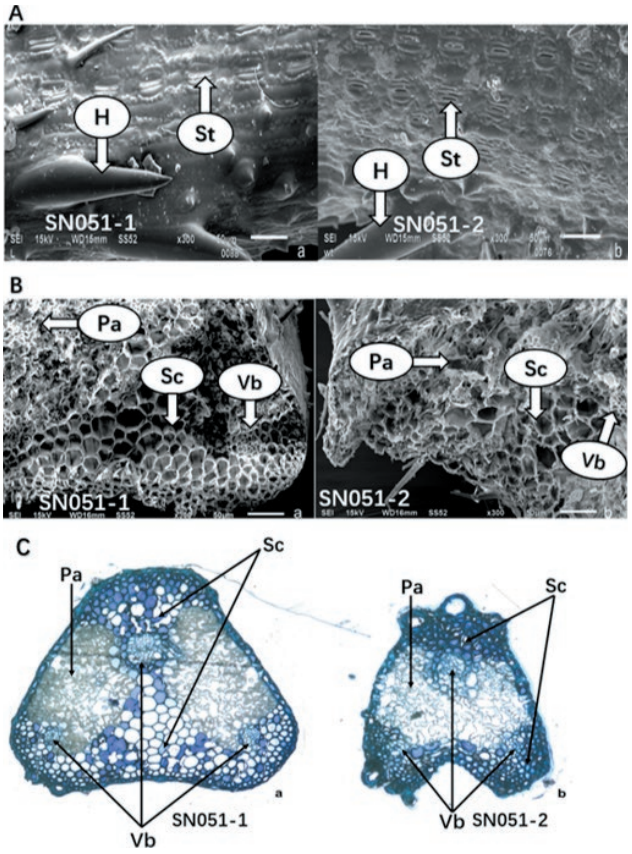

Figure 4. Awn external surface and cross-section of NILs. A. SEM images of the awn epidermis. St: stomata; H: hair. a. SEM images of the epidermis of SN051-1 awn, b. SEM images of the epidermis of the SN051-2 awn. B SEM images of the morpho-anatomical structure of awns. Vb: vascular bundle; Sc: sclerenchyma; Pa: parenchyma an SEM image of the morpho-anatomical structure of SN051-1 awn b SEM images of the morpho-anatomical structure of the SN051-2 awn. C. Morpho-anatomical structure of awns by semi-thin sections. Vb: vascular bundle; Sc: sclerenchyma; Pa: parenchyma.

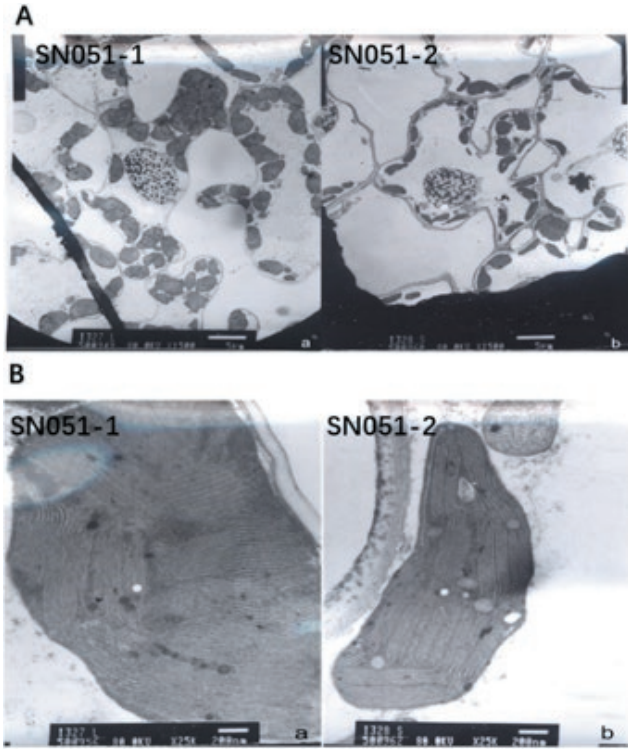

Figure 5. Ultrastructure and TEM results of chloroplast. A. Ultrastructure of chloroplast in SN051-1 awn cells b ultrastructure of chloroplast in SN051-1awn cells. The chloroplast in SN051-1 awn cells was bigger and more than that in SN051-2. B. TEM images show the ultrastructure of a single chloroplast in awns. An ultrastructure of single chloroplast in SN051-1 awn cell b ultrastructure of single chloroplast in SN051-1 awn cell. The chloroplast in SN051-1 awn cell was bigger than that in SN051-2.

are similar to those in leaves and well developed (Figure 5A). SN051-1 had more chloroplasts in each cell (Figure 5A), and the volume of the chloroplast of SN051-1 awn cell was significantly bigger than that in SN051-2 cells (Figure 5B). Besides, because the long awn has a larger surface area than the short one, it means that it has more chloroplasts, suggesting that wheat lines with long awn had a better capacity of photosynthetic than short awn plants.

Differences in photosynthetic rate and grout rate and ear in the near-isogenic lines (NILs) with the difference of awn-length

The photosynthetic rates of flag leaves and the panicle of SN051-1 and SN051-2 were measured 27 days after flowering (Figure 6). It can be seen that there was no

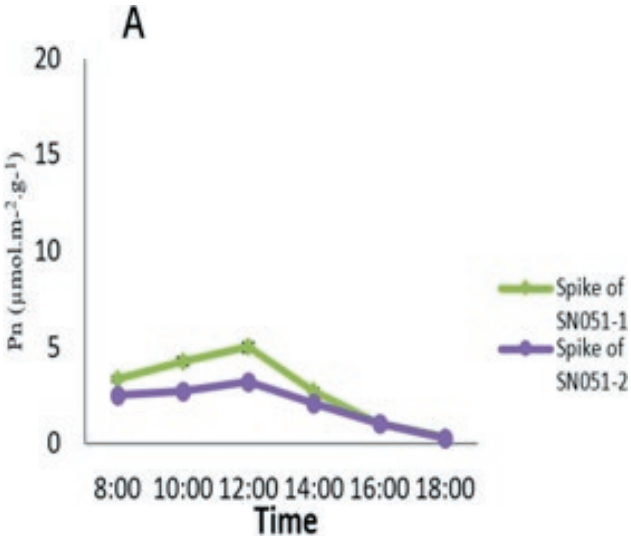

Figure 6. Curve of the daily net photosynthetic rate of flag leaf for SN051-1 and SN051-2.

Table 2. the thousand seed weight of long awn plants and short awn plants.

Materials	Awns	Average TKW	F Value
(SN051-1/SN051-2) F2	Long awn	32.396	8.637**
	Short awn	30.778	
(SN051-2/SN051-1//SN051-1) BC1	Long awn	32.709	5.146*
	Short awn	30.743	

obvious midday depression in a panicle; the panicle net photosynthetic rate of SN051-1 was significantly higher than that of SN051-2, and the difference reached a significant level. This result indicated that the presence of awn significantly increased the photosynthetic capacity of the panicle.

The comparison of thousand-grain weight per line in F_2 generation between long awn plant and short awn indicated that thousand kernels weight with long awn is higher than that without an awn (Table 2). This result suggested that awn played an important role in the accumulation of assimilates and photosynthesis in the later stage of grain filling, which resulted in the difference of thousand-grain weight between long awn plants and short awn plants.

DISCUSSION

Awn-inhibitor gene B1

In common wheat, *B1* (5AL), *B2* (4AS), and *Hd* (6BL) are known dominant suppressor genes of awn, and different combinations of them can lead to changes in awn phenotype (Sourdille et al., 2004; Mackay et al., 2014). Homozygotes of three recessive alleles *b1*, *b2,* and *hd* were awned phenotypes, the presence of one dominant allele inhibited the elongation of awn, and the plants containing two dominant inhibitory alleles were short awn phenotypes (Antonyuk et al., 2012). Previous studies have shown that the effect of the wheat *B1* gene on the length of awn is the strongest, results in very short or absent awns at the base and the middle of the ear. (Yoshioka et al., 2017; DeWitt et al., 2020; Huang et al., 2020). So far, although the *B1* gene has been cloned, the mutation site and function of the *B1* gene are still controversial in previous researches, and further experimental verification is needed, and its expression and inhibition mechanism are still a mystery.

Compared with other photosynthetic organs, awn has several advantaged conditions for photosynthesis (Wang et al., 1993). The presence of awns can double the rate of net ear photosynthesis (Evans and Rawson, 1970), it contributed about 40-80% of the total spike carbon exchange rate, depending on the species (Grundbacher 1963; Das and Mukherjee 1991; Blum et al. 1985; Khaliq et al. 2008; Motzo and Giunta 2002). Statistical analysis found that spikelet number of short awn individuals was higher than that of long awn individuals, but the grain weight and bulk density of long awn individuals increased by 6.3% and 11.6% compared with that of short awn individuals. The previous results showed that the *B1* gene was significantly correlated with spikelet number, grain weight, and bulk density of wheat (DeWitt et al. 2020). However, its regulation mechanism is not clear.

The agronomic characters of SN051-1 (long awn) and SN051-2 (short awn) in this study were same, and other characters, such as anthesis date, plant height, spike length, spike number per plant and kernels per spike, were also similar, only the length of awn and grain weight were different (Du et al. 2010). The results of polymorphic primers also indicate that the genetic backgrounds of the two near-isogenic lines are highly consistent. The results of molecular markers and character analysis of awn showed that SN051-1 (long awn) and SN051-2 (short awn) were a pair of the proximal isogenic lines with awn length suppressor gene *B1* on 5AL chromosomes. SN051-1 (long awn) and SN051-2 (short awn), this excellent experimental material laid a good material foundation for the study of the role of awn and the mechanism of *B1* gene expression and inhibition.

In the present study, we applied SEM to observe long awn microstructure and found wheat long awn possessed typical xerophytes structure, which is possibly associated with high adaptation on the special region. It was reported that the proportion of awned varieties has increased over the past two decades in warm growing regions such as the southeastern U.S (Motzo & Giunta, 2002). Furthermore, the awn stomatal density of long awn Line SN051-1 with gene *b1* was less than Line SN051-2, while the awn cross-sectional area, cell volume, and volume ratio of the chloroplast of Line SN051-1 were far larger than Line SN051-2. These characteristics indicated that long awn lines SN051-1 had a stronger photosynthetic capacity, which can contribute to large grain and high grain yield in long awn wheat cultivars, particularly during the grain-filling stages. Moreover, the thousand-grain weight of the long awn NIL-*b1* SN051-1 is higher than that of short awn NIL-*B1* SN051-2. It provides cell structure evidence that awn is the major photosynthetic organ of the spike. In barley, a recent study showed that the awn preferentially expressed genes for photosynthesis, the biosynthesis of chlorophyll and

carotenoids, and reactive oxygen species scavenging, while the lemma and palea overexpressed defense-related genes compared with the awn (Abebe et al. 2009). The results suggests the lemma and palea are mainly protective organs, whereas the awn is primarily a photosynthetic organ. Therefore, molecular evidence that wheat awn is the major photosynthetic organ of the spike is still needed.

CONCLUSION

In this study, SN051-1 (long awns) and SN051-2 (short awns) derived from same cross combination showed morphological traits but awn length difference, and similar genetic background but different Xgwm291 genotype, a linkage marker with Gene *B1*, which can be regarded as near-isogenic lines (NILs) with different awn length. Besides the awn length trait, we further investigated awn cell structure and yield-related traits of the NILs. The results showed there were significant differences between awn anatomy, the photosynthetic rate, thousand kernel weight. The NIL-*b1* line, SN051-1, had long awn, which is more conducive to the cell structure of photosynthesis.

AUTHORS' CONTRIBUTIONS

Conceived and designed the experiments: FQ and XL. Performed the experiments: FQ, YZ. Manuscript preparation: FQ and XL. All authors read and approved the final manuscript.

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Evaluation of the genotoxicity of some standart and eco-friendly detergents with *Vicia faba*

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Abstract. The increasing use of detergents as a result of rapid increase of population and irregular industrialization has a big role in the environmental pollution. Determination of genotoxic effects of some standart and eco-friendly detergents on *Vicia faba* was aimed in this study. Faba beans were treated with detergents for 24, 48 and 72h by using EC50 and 2xEC50 values and analysed by mitotic index (MI) and comet assay. According to the results of root inhibition test, EC50 value for the detergent A1 was 20ml/L, A2 was 50ml/L, B1 was 40ml/L, B2 was 60ml/L respectively. The least decrease in the mitotic index was found in detergent B2 (eco-friendly laundry) and the most decrease was found in detergent A1 (standart dishwasher). Various abnormalities were determined in different phases of mitosis. After comet assay the cells were classified as type 0, type 1, type 2, type 3 and type 4 according to the nuclei damage rates. Type 3 was the most identified cell damage degree in eco-friendly detergents whereas type 4 was the mostly seen in standard detergents. Parameters such as olive tail moment, tail DNA percentage and arbitrary units were also identified by comet assay. All the test results showed that standard detergents cause more genotoxic effects than eco-friendly ones.

Keywords: Comet assay, detergents, DNA damage, chromosome aberations, mitotic index, *Vicia faba*.

INTRODUCTION

Hundreds of chemicals continue to be produced to make our lives easier. Although they are useful in raising our standard of living, we cannot understand the fate of these chemicals without investigating their effects on the living things. Plant biotests have an important role in determining the cytotoxic and genotoxic effects of environmental pollutants, chromosome damage and gene mutations. These studies can contribute to the creation of a database about environmental conditions in various regions of the world. The simple and clear results of the biotests can also be used in public awareness training on the genotoxic effects of environmental pollution.

Recently, pollution from domestic and industrial wastes is increasing and detergents are replaced by a large percentage of effective pollutants (Minareci

ve diğ., 2008). Detergents are mixtures of powder, granule, soft consistency or liquid form, which are preferred for cleaning purposes and which contain anionic surfactants and other cleaning agents as primary cleansers (Egemen, 2000). Laundary detergents are produced in large quantities and used in daily life and industrial activities (Wang et al., 2019). Various physical and chemical analyzes are carried out to determine environmental pollution of the detergents, however, these analyzes alone are not sufficient. Genotoxicity studies are essential to determine the effects of detergents on organisms.

Many plants have been used for cytotoxicity testing, recent reports being of *Allium cepa* L. (Bonciu et al., 2018), *Vicia faba* L., *Zea mays* (Bonea and Bonciu, 2017), and *Drimia indica* (Roxb.) Jessop. (Daphedar and Taranath, 2018). *V.faba* root tips, can be easily obtained in a whole year, its development and storage is easy and inexpensive, the root meristem comprises a high proportion of cells undergoing mitosis and the number of chromosomes is low (2n = 12) and appropriate for accurate and complete counting. The *V.faba* root meristem chromosomal mutation assay is also approved by the International Programme on Chemical Safety (IPCS, WHO) and the United Nations Environment Programme (UNEP) as an effective and standard test for the chemical screening (Youssef and Elamawi, 2020).

The most important point in the root growth inhibition test is the determination of the effective concentration (EC50) which reduces the root length by 50% compared to the control group (Fiskesjö, 1985). In order to determine the toxic and genotoxic effects of environmental pollutants on chromosomes and cell division EC50 values were calculated in many studies (Rank and Nielsen, 1998; Chauhan et al., 1999, Ateeq et al., 2002; Saxena et al., 2005; Arıkan, 2006).

Mitotic activity tests are frequently used in chromosome counting and toxicological research. By the application of this test, information can be provided about the effect of chemical treatment on cell division (Akı and Karabay, 2004).

Cytogenetic methods are widely used in the biological monitoring of populations exposed to mutagenic and carcinogenic compounds. To date, many methods have been used to detect DNA damage. One of them is comet analysis that is widely used because it is a sensitive, fast and reliable method for the detection of various types of DNA damages.

In addition to visual assessment of DNA damage by comet technique, parameters related to tail length, tail moment, and percentage of DNA in the tail can be determined using comet image analysis programs (Collins, 2002). High toxicity may be the cause of the increase in DNA damage. In a study on the genotoxic effects of pollutants in some plants, a relationship between concentration and DNA damage has been demonstrated (Gichner et al., 2006).

There are a few studies that have evaluated the toxicity and genotoxicity of commercial eco-friendly detergents in plant bioassays, their toxic effects assessed generally in *Daphnia magna* (Pettersson et al., 2000), some microalgae (Aizdaicher and Markina, 2006; Azizullah et al., 2011), in *Salmonella* test and in human leukocytes (Pedrazzani et al., 2012).

It was aimed to determine the genotoxic effects of four different dishwasher and laundry detergents, two of which were eco-friendly, on *V. faba* using the root-tip chromosome aberration test of accepted plant biotests. Mitotic index and mitotic abnormalities were investigated with cytotoxicity test and DNA damage was investigated with the single cell gel electrophoresis (SCGE) method which is also called as comet assay. Thus, it will provide new sights to the environmental pollution and indirectly find out the damage caused to the other organisms via the food chain.

MATERIALS AND METHODS

V. faba (2n = 12) seeds were used as research material obtained from local market. The commercial dishwasher (A1 and A2) and laundry (B1 and B2) detergents, two of which were standart named as A1 and B1 and two of which were eco-friendly named as A2 and B2, were used. Chemical content information about detergents was shown in Table 1.

Germination of V. faba seeds and determination of EC50 concentrations

Surface sterilization was performed by selecting dimensionally and morphologically uniform *V. faba* seeds. The seeds were shaken in 70% alcohol for 1 minute, after 15 minutes in a 3% sodium hypochlorite containing 2 drops of Tween-20 for 200 mL, and then washed 4 times with sterile distilled water. Sterilized seeds were kept in sterile distilled water for 12 hours to swell before they were germinated (Hamdy and Hattori, 2006).

Petri dishes used to germinate the seeds were first sterilized in autoclave and then exposed to UV light in a sterile cabinet for 15 minutes. Sterile Whatman papers treated with detergent concentrations were placed into sterile petri dishes placed at regular intervals. 20 pieces of seed were placed in each petri and covered with

Table 1. The chemical contents of the four detergents A1, A2, B1 and B2.

Detergents	Chemical content
A1	15-30% Anionic active substance, Polycarboxylate, Phosphonate, Enzyme (Amylase, Protease), Perfume, Paint, Preservative.
A2	<5% Soap, <5-15 Anionic Substances, <5% Nonionic Substances, Perfumes, Methylisothiazolinone, Water, Raw Materials from Coconut, Palm, Wheat and Potato.
B1	% 5-15 Anionic active substance, Nonionic active ingredient, <5% Phosphonate, Soap, Enzyme, Perfume (Alpha Ionone, Amyl Cinnamal, Benzyl Salicylate, Butylphenyl Methylpropional, Hexyl Cinnamal, Limonene, Linalool), Preservative.
B2	<5% Soap, <5-15 Anionic, <5% Nonionic Substances, Perfume, Methylchloroisothiazolinone, Water, Raw Materials from Coconut, Palm, Wheat and Potato.

A1 and B1 are standard detergents. A2 and B2 are classified as eco-friendly detergents.

Whatman papers treated with detergent concentrations and then put into 24 °C incubator.

The detergent concentrations used in determination of EC50 values for each detergent was listed in Table 2. The root length of the seeds, which were germinated in the incubator for 7 days, were measured for root inhibition test. At the end of one week, the averages of the root lengths were calculated and the decrease in the root lengths were compared with the control group. The test set-ups for calculating the EC50 value were performed in 3 replicates. After the EC50 and 2xEC50 values were calculated, all experiments were carried out on these values.

Table 2. The detergent concentrations used in determination of EC50 values.

Detergent	Concentration (ml/L)	Detergent	Concentration (ml/L)
A1	1	B1	1
	10		10
	20		20
	30		30
	40		40
	50		50
	100		100
	200		200
	400		400
A2	1	B2	1
	10		10
	50		50
	100		60
	200		70
	400		80
			90
			100
			200
			400

Chromosomal abberation test

At the end of 24, 48 and 72 hours, the roots that were approximately 1-2 cm long were cut with the help of sterile scalpel and transferred to Carnoy (3 parts 70 % alcohol, 1 part 45 % glacial acetic acid) fixation fluid. After 24 hours, the root tips were put in 70 % ethyl alcohol and then hydrolised in 1 mL 1 N HCl with the help of the burner flame. The roots that were hydrolyzed in the watch glass were stained with 1-2 drops of 2 % aceto orsein (Merck, Germany). The cover glass was covered on the stained root tip and the crush-smear preparation technique was applied. Photographs of the cells were taken with 40x and 100x lenses to determine chromosome abnormalities with Olympus BX51 photomicroscope. In the calculation of the percentage of mitotic index, 500 cells were counted in each of the 10 slides were prepared for each concentration and 5000 cells were counted for each concentration (Yüksel and Aksoy, 2017). The percentage of mitotic indices was then determined by dividing the number of cells divided by the total cell number and multiplying by 100 for each application concentration (EC50 and 2xEC50), duration (24, 48, 72 h) and their control groups (Souza et al., 2013). The ratios of chromosomal damage such as chromosome bridge, chromosome adhesion, pole shift and laggard chromosome formation in dividing cells were calculated.

SCGE (Single Cell Gel Electrophoresis) Analysis

400 µL cold tris buffer solution and 5-6 root tips (1-2 cm) of *V.faba* were put in petri dishes placed on ice cassettes. The roots were cut gently with the aid of a scalpel until the color of the buffer solution was blurred without taking over the ice cassettes. The resulting suspension was transferred to the ependorf tube and left on ice for 15 minutes to allow the nuclei of the meristematic cells to settle down. The slides were coated with 1% NMP (normal melting point) agarose (ROTH Germany 2267, Sigma, A9539). 100 µL of the nuclei solution was taken close to the bottom of the ependorf tube, and 100 µL

of LMP (low melting point) agarose (ROTH Germany, 6351, Sigma, A9414) was mixed by pipetting in ependorf tube, allowing the nuclei to adhere to the LMP agarose. Nuclei + LMP agarose mixture was poured onto NMP coated slides and closed with lamella immediately. The slides were kept on ice for 10 minutes at +4°C in the refrigerator to stabilize the agarose. Cover glass on the slides were gently removed after taken from the refrigerator. The slides were placed in the same direction and adjacent to each other in the horizontal dark electrophoresis tank, which had been pre-cooled to +4°C with the cold water bath.

A cold SCGE buffer was poured slowly from the edges of the tank and the cover of the tank was closed. The slides were stored in the tank for 20 minutes in order to dissolve the DNA chain. Electrophoresis (CSL-COM 20, 1000 mL, and Cleaver CS-300V power supply) was performed at 27V, 300mA for 25 minutes in order to observe the comets that would occur as a result of different molecular weight DNA fragments. At the end of the electrophoresis, the slides were removed from the tank and placed in the trays and kept in cold tris-HCl 3 times each for 5 minutes for neutralization.

After the neutralization process, the slides were arranged upright on the drying paper and the excess buffer was allowed to move over the slides. The slides were stained with 100 µL of Red Safe dye before drying and left for 5 minutes. They were closed immediately with cover glass. Slides for comet assay were examined by fluorescence microscope Olympus BX51. For each concentration and time, 25 randomly chosen nuclei were analyzed and three slides were evaluated per treatment and the median values of comet parameters for each slide were calculated (Türkoğlu, 2012). The averaged median tail length (µm), percentage of tail DNA (% of DNA in comet tail), olive tail moment (OTM in arbitrary unit) values were calculated using the Kameram Software (Argenit, Turkey) comet assay analysis program for each treatment group.

RESULTS

As a result of the calculations made respectively; EC50 for A1 was determined as 20 ml/L (Fig 1a) and 2xEC50 was 40 ml/L; EC50 for A2 was determined as 50 ml/L (Fig 1b) and 2xEC50 was 100 ml/L; EC50 for B1 was determined as 40 ml/L (Fig 1c) and 2xEC50 was 80 ml/L; EC50 for B2 was determined as 60 ml/L (Fig 1d) and 2xEC50 was 120 ml/L.

It was observed that the percentage of root lengths decreased gradually compared to control in increasing detergent concentrations. It was also observed that the EC50 values of the eco-friendly detergents were higher than the standard detergents (Fig. 1).

The effects of the detergents A1, A2, B1, B2 on mitotic frequency and chromosomal abnormality of V.faba root tip cells

The mitotic index values were decreased in the treatment groups due to increasing time and concentration at the end of 72 hours. Mitotic index values of eco-friendly detergents were higher than the standard detergents (Table 3).

At the end of the seven days, the mitotic index value for the control group was found to be 14.60%, it was 8,90% at 20ml/L and 7,80% at 40ml/L A1 detergent concentration; the mitotic index value for A2 detergent, decreased to 12.80% in 50 ml/L and to 11.50% at 100ml/L concentration; the mitotic index value for B1 detergent, decreased to 8.30% in 40 ml/L concentration and to 8.10% at 80 ml/L concentration; the mitotic index value for B2 detergent, decreased to 11.90% in 50 ml/L concentration and to 10.60% at 100ml/L concentration.

The observed chromosomal abnormalities for all of the detergent treatments of *V.faba* root tip cells were; laggard chromosome, chromosome adhesion, irregular anaphase, chromosomal bridge, chromosome swelling, micronucleus formation, pole shift, C-mitosis, irregular prophase, irregular metaphase and chromosome stickness (Fig 2).

The detection of DNA damage caused by detergents with SCGE

The degree of DNA damage increased in proportion to the concentration and duration, and the degree of DNA damage in the eco-friendly detergents was lower than the standard detergents. The classification of nuclei according to the degree of damage with SCGE yield five types of nucleus; “type 0” means no visible tail, “type 1” means tiny tail , “type 2” means dim tail, “type 3” means a clear tail that is longer than the head diameter and “type 4” means tail is approxiametly three times longer than the head (Fig 3).

In the control groups, a few DNA damaged nuclei, called type 0 and type 1, were observed.

Type 1 and type 2 grade DNA damages were observed at 20ml/L A1 concentration after 24 and 48 hours treatment, and type 3 grade DNA damage was observed to occur more than type 2 after 72 hours. Type 2 grade DNA damages were seen mostly in 24 hours at

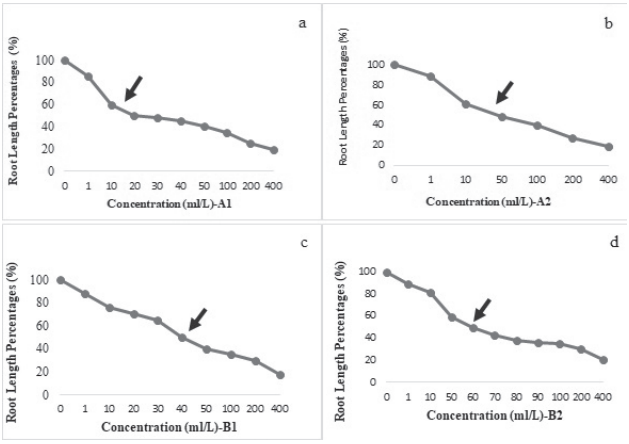

Figure 1. EC50 values determined for the four different detergents, a. detergent A1, b. detergent A2, c. detergent B1, d. detergent B2. Arrows show the EC50 values.

Table 3. Mitotic index percentages calculated for the detergents A1, A2, B1 and B2 after 72 h. treatment.

Detergent	Concentration(ml/L)	MI (%) ± SD*
A1	0	14.60±0.07
	20	8.90±0.08
	40	7.80±0.09
A2	50	12.80±0.11
	100	11.50±0.07
B1	40	8.30±0.09
	80	8.10±0.05
B2	60	11.90±0.04
	120	10.60±0.05

* MI ± SD: average mitotic index percentage±standart deviation.

a concentration of 40 ml/L A1, while type 2 and type 3 grade damages were observed at 48 hours. Type 3 and type 4 grade damages were observed in majority after 72 hours.

Type 0 and type 1 grade DNA damages were detected at 50 ml/L A2 concentration treated for 24 hours. Type 1 and type 2 grade DNA damages were found at the end of 48 hours, and type 3 grade DNA damage was observed when 72 hours were completed. Type 0 and type 1 grade DNA damages were detected at a concentration of 100 ml/L for 24 hours treatment. Type 2 grade DNA damages were observed frequently during 48 hours, and nuclei with DNA damages in type 2 and type 3 were observed at the end of 72 hours.

Type 1 and type 2 grade DNA damages were observed at 40 ml/L B1concentration for 24 hours, while nuclei with type 2 grade DNA damage were observed after 48 hours treatment. Type 3 grade DNA damage was seen after 72 hours. Type 2 grade DNA damage was found in 80 ml/L concentration after 24 hours and type 2 and type 3 grade damage was found after 48 hours treatment. At the end of 72 hours, nuclei with type 4 grade DNA damage were observed. Type 0 and type 1 grade DNA damages were determined at 60ml/L B2 concentration treated for 24 hours. Type 2 grade DNA damage was observed after 48 hours and DNA damage level changed to type 3 after 72 hours. Type 1 and type 2 grade DNA damages were observed at 120 ml/L concentration for 24 hours and type 2 grade DNA damage was observed after 48 hours. Type 3 grade DNA damages were determined after 72 hours.

The effects of different concentrations of detergents on DNA percentage (tail DNA%) were also examined. Tail DNA percentages for the control group of A1 detergent was 2.25% after 24 hours, 3.6% after 48 hours and 4.2% after 72 hours. At a concentration of 20 ml/L A1 detergent, the value increased from 22.4% after 24 hours to 43.6% in 48 hours, reaching 51.8% at the end of 72 hours. Tail DNA damage, which was 40.3% at 40 ml/L concentration in 24 hours, reached 52.3% in 48 hours and 66.7% in 72 hours (Table 5).

The tail DNA percentages was 0.9% after 24 hours in the control group of A2 detergent, 0.7% at 48 hours and 1.5% at 72 hours. At a concentration of 50 ml/L, the value of 17.2% in 24 hours increased to 22.9% in 48 hours. At a concentration of 100 ml/L, it was observed that the value of 29.4% in 24 hours, 38.1% in 48 hours and 49.2% in 72 hours (Table 5).

The tail DNA percentages was 1.8% in the control group of B1 detergent after 24 hours which was, reached 2.3% in 48 hours and 2.6% in 72 hours. At a 40 ml/L B1 detergent concentration, the value of tail DNA was 26.4% at 24 hours, 40.7% at 48 hours, and 50.5% at 72 hours, At a concentration of 80 ml/L, it was observed that the value was 42.6% in 24 hours reached 62.8% in 48 hours and 66.1% in 72 hours (Table 5).

The tail DNA percentages was 0.65% for B2 detergent in the control group of B1 detergent after 24 hours which was reached 0.8 % in 48 hours and 1.27% in 72 hours. At a concentration of 60 ml/L, the value of 18.5% at 24 hours reached 25.9% at 48 hours, and at 72 hours the value of tail DNA was 42.4%. At a concentration of 120 ml/L, it was observed that the value was 32.6% in 24 hours, 46.4% in 48 hours and 50.8% in 72 hours (Table 5).

For all detergent treatments on *V. faba*, the values of the tail were calculated as well as the determination of the percentage of tail DNA that increased in direct proportion to the concentration and duration.

The effect of all detergents on the OTM value at varying concentrations is shown in Table 6.

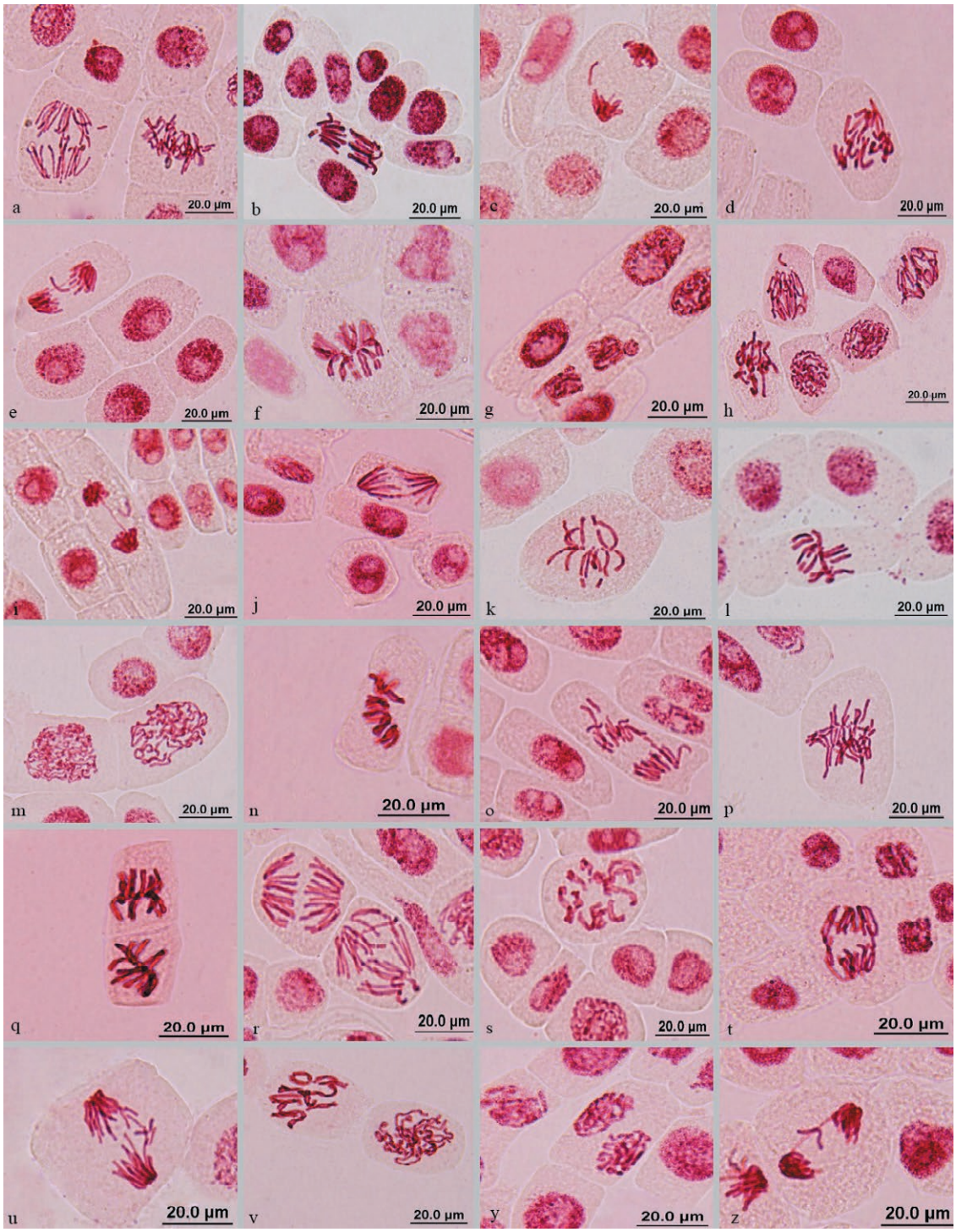

Figure 2. The chromosomal abnormalities caused by detergents in V. faba meristematic cells a. Laggard chromosome in anaphase and chromosome adhesion in metaphase, b. Laggard chromosome in anaphase, c. Laggard chromosome in telophase, d. Irregular anaphase, e. Chromosomal bridge and laggard chromosome in anaphase, f. Chromosome swelling in metaphase, g. Micronucleus formation in cytokinesis, h. Laggard chromosome and chromosome adhesion in anaphase, i. Chromosomal bridge and laggard chromosome in telophase, j. Chromosomal bridge and pole shift in anaphase, k. C-mitosis, l. Laggard chromosome in metaphase, m. Irregular prophase, n. Pole shift and chromosome swelling in metaphase, o. Laggard chromosome in anaphase, p. Irregular metaphase, q. Pole shift in metaphase, r. Irregular anaphase, s. C-mitosis and chromosome stickness, t. Chromosomal bridge in anaphase, u. Laggard chromosome in anaphase, v. C-mitosis and irregular prophase, y. Pole shift in telophase, z. Chromosomal bridge and laggard chromosome in telophase.

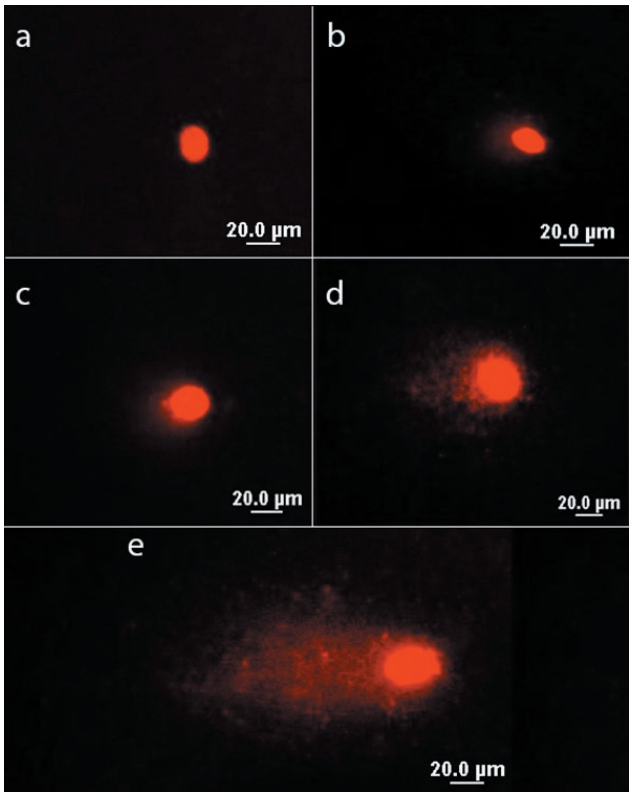

Figure 3. Classification of nuclei according to the degree of damage in *V.faba* with Comet test a) Type 0: undamaged, b) Type 1: tail <0,5 c) Type 2: tail>0,5 or equal to the head, d) Type 3: tail is longer than the head diameter, e) Type 4: tail is approxiametly three times longer than the head.

OTM values at all detergents showed a significant increase due to time and concentrations.

Table 4. The effect of the detergents on chromosomal abnormality percentages in *V.faba*

Detergent	Concentration (mg/L)	Duration time(h)	Chromosomal abnormality(%)±SD*
A1	0	24	0.25±0.27
		48	1.70±0.48
		72	2.70±0.66
	20	24	8.0±0.05
		48	11.0±0.07
		72	14.0±0.07
	40	24	23.2±0.06
		48	26.2±0.07
		72	30.4±0.07
A2	50	24	7.7±0.07
		48	9.2±0.05
		72	8.9±0.54
	100	24	11.8±0.06
		48	13.7±0.49
		72	14.1±0.07
B1	40	24	9.6±0.07
		48	12.2±0.54
		72	15.6±0.06
	80	24	21.1±0.07
		48	32,7±0.05
		72	28.3±0.06
B2	60	24	6.6±0.06
		48	8.3±0.05
		72	9.9±0.06
	120	24	10.8±0.05
		48	12.7±0.07
		72	16.5±0.07

DISCUSSION

V. faba seeds treated with detergents were allowed to grow and the root inhibition test and the values of the root length halving (EC50) by the end of seven days compared to the control group were determined for each detergent separately. It was found that the standard detergents could reduce the root length in lower concentrations than the eco-friendly detergents. Studies on the plant assays revealed that any genotoxic effects manifested in a test sample are likely to result in inhibition of root growth (Yekeen et al. 2017).

Ezemonye et al. investigated the genotoxicity of high anionic surfactant-containing detergents on sweet and salt water shrimps (*Desmoscaris trispinosa* and *Palaemonetes africanus*), it has been shown that increasing detergent concentrations increase the mortality rate compared to the control group (Ezemonye et al., 2009). Wing et al. (2019) demonstrated that both laundry detergents and detergent residue after rinsing showed high cytotoxicity on human bronchial epithelial cell culture.

Pettersson et al. found out the toxic effects of 26 laundry detergents and 5 softeners on *Daphnia magna*, it was reported that the concentration of detergents and the genotoxic effects were correlated with each other (Pettersson et al., 2000). The cytotoxic/genotoxic effects (by mitotic index and micronuclei frequency) of Boron used as detergent additive were reported in root meristems of *V. faba* (Barbefieri, 2016).

The lowest EC50 value was determined in A1 standart detergent that its effective concentration was found as 20 ml/L. According to this finding, A1 standart detergent is the most toxic from all of the other studied detergents and gave damage to the root mitotic cells in

Table 5. The effect of the detergents on Tail DNA percentages in *V. faba*

Detergent	Concentration (ml/L)	Duration Time/ DNA% in tail		
		24 h	48 h	72 h
A1	0	2.25	3.60	4.20
	20	22.40	43.60	51.80
	40	40.30	52.30	66.70
A2	0	0.90	0.70	1.50
	50	17.20	22.90	37.80
	100	29.40	38.10	49.80
B1	0	1.80	2.30	2.60
	40	26.40	40.70	50.50
	80	42.60	62.80	66.10
B2	0	0.65	0.80	1.27
	60	18.50	25.90	42.40
	120	32.60	46.40	50.80

Table 6. The effect of the detergents on OTM values in *V. faba.*

Detergent	Concentration (ml/L)	Duration Time/ OTM value		
		24h	48h	72h
A1	0	0.75	1.2	1.4
	20	7.5	16	17.2
	40	14.7	17.5	22.2
A2	0	0.2	0.3	0.57
	50	6	8.6	12.6
	100	10.8	12.7	16.6
B1	0	0.63	0.8	0.9
	40	8.8	14.3	17
	80	15.2	20.9	22.3
B2	0	0.21	0.26	0.6
	60	7.17	8.7	14.1
	120	11.3	15.4	17

V. faba. The highest EC50 value was determined in B2 eco-friendly detergent that its effective concentration was found as 60 ml/L. Therefore, the lowest amount of damage to root mitotic cells in *V. faba* was caused by B2 detergent. As a result, the chemicals in standard detergents have negative effects on root growth in *V. faba* compared to the chemicals in eco-friendly detergents.

Mitotic index test results showed that there was a reduction in mitoic index percentage with increasing time and concentration in all detergents. The reduction in mitotic index can be explained by the mitodepressive effects of chemicals in detergents, causing errors in normal cell cycle and limiting cell division (Özkara et al., 2015).

A1 detergent was the detergent that decreased the mitotic index percentage by 10.9% compared to the control, while B2 detergent was the detergent that decreased the mitotic index by 2.4% compared to the control. The mean percentage value of mitotic index was higher in the eco-friendly detergents than the standart detergents. It was concluded that the standard detergents influence cell division negatively when the chromosomal abnormalities in the root tip meristematic cells of *V.faba* were determined. Chromosomal abnormalities occur as a result of inability to repair the fractures in DNA double chain (Maluszynska and Juchimiuk, 2005).

In our study, it was determined that chromosomal aberration rate increased significantly in all detergents due to increasing concentration and duration. The most common abnormalies were polar shift, irregular prophase, irregular metaphase, sticky chromosome, c-mitosis and bridge formation. Induction of chromosomal aberrations pointed to potential for genotoxicity (Yekeen et al., 2017). Wang et al. (2019) reported that due to the ability of the detergents that break the lipid-lipid and protein-lipid interactions, membrane proteins and lipids can become soluble in human bronchial epithelial monolayer cell cultures. However, in our study, such a change was not observed visually due to the cell wall in plants but may be observed with cell ultrastructure studies.

The highest chromosomal abnormality was observed in A1 detergent at a rate of 30.4% after a period of 72 hours while the lowest chromosomal abnormality was observed in A2 detergent with a value of 14.1%. The standard detergents were found to have more chromosomal abnormalities than eco-friendly detergents. The chromosomal aberrations observed in this assay suggest that all the detergents exert a mutagenic/cytotoxic effect.

A wide variety of DNA repair mechanisms are available to prevent such damage in the cell nucleus. Replication, transcription and protein synthesis inhibition may occur when these repair mechanisms are ineffective or when very severe DNA damage occurs. However, chromosomal abnormalities and mutations can be seen in the long term treatments (Aksoy, 2017).

The damage caused by the genotoxic effects of detergents was also investigated by comet assay test. The damages were classified in five groups as type 0, type 1, type 2, type 3 and type 4 according to their degree of damage. Type 3 DNA damage was mostly seen in standard detergents at the highest concentrations and durations.

In A1 and B1 detergents, DNA damage from the head of the comet tail reached almost threefold, while eco-friendly A2 and B2 detergents showed DNA damage

of up to twice the size of the tail. As a result, the chemicals in the standard detergents caused much more damage on the DNA of *V.faba* root tip cells than eco-friendly detergents. A1 is the standart detergent that reaches the most intense tail DNA percentage while the A2 eco-friendly detergent has the lowest concentration when treated with the highest concentration and time period. Studies have also shown DNA fragmentation in *V. faba* root apical meristem cells and seedlings exposed to toxic compounds with the highest concentrations and different time periods (Arya et al., 2013; Liu et al., 2015; Ghosh et al., 2016, Iqbal, 2016; Hu et al., 2017; Cortés-Eslava et al., 2018; Youssef and Elamawi 2020).

As a result, it was determined that the percentage of tail DNA in increasing concentrations and durations was higher in standard detergents compared to eco-friendly detergents. The standard detergents caused more genetic damage because they produced more dense DNA-containing tails. As in the tail DNA percentage, olive tail moment (OTM) values showed similar results in terms of increasing concentration and duration. DNA tail percentage was studied with OTM parameters in a another study on the determination of genetic damage on onion root tips treated with 5-100% concentrations of coal ash (Chakraborty et al., 2009). OTM and the percentage of tail DNA correlate well with the concentrations of chemical substances with genotoxic effect and are parameters that give confidence in comet assay evaluations (Kumaravel et al., 2009). In our study, B1 standart detergent had the highest OTM value while the A2 eco-friendly detergent was the lowest. As a result OTM value of eco-friendly detergents remained lower than standard detergents.

Sobrino-Figueroa stated that since detergents are complex mixtures of different substances, in which additive and/or synergistic effects may occur, the deleterious effect caused by the dishwasher detergent was probably due to the combined effects of the ingredients of the detergent. (Sobrino-Figueroa, 2013).

When all results obtained in the study were evaluated in general, it was observed that eco-friendly detergents produced significantly less genotoxic effects on *V.faba* root tip cells than standard detergents. This is due to the fact that in the production of eco-friendly detergents that we have chosen to use in the study, a lower proportion of anionic material and nonionic material is used than standard detergents. In addition, the use of raw materials from coconut, palm, wheat and potatoes in production to reduce the proportion of chemicals in its content has also been a supportive factor in creating less genotoxic damage on living beings.

The results obtained in this study indicate that detergent wastes that reach foodstuffs through food chain cause serious damage to DNA. In light of all these findings, it is obvious that there are serious measures to be taken at the stage of detergent production and use.

Detergents should be treated more carefully in their production due to the negative effects on the life, development and genetics of living things. Detergents that cannot be sufficiently rinsed due to the use of more detergents than necessary are the main reasons that cause residual residues in washing dishes and laundry to cause direct health problems. As it provides only pleasant odor or softness, it should be more sensitive to the consumption of detergents used in addition to cleaning. The amount of waste detergent to be left to the environment should not be increased for such reasons, which is not necessary. Being conscious of consumption and having the consciousness of protecting the environment is extremely important for us to leave a cleaner environment for future generations.

In addition to the measures that consumers can take, there are some points that manufacturers should take into consideration. The products should be subjected to the necessary tests at every stage of production before they are put on sale. The studies on the determination of the harmful effects of detergents on the environment and the environment and the studies to reduce these damages should be made on the basis of the production of raw materials. Particularly in the case of detergents, the amount of phosphate used must be limited and the amounts involved in the water must be removed in phosphate treatment plants. The capacities of the treatment facilities should be increased in order to be able to make a healthier treatment. Any industrialization without taking measures to protect nature should be prevented. Drinking and operating water must be checked periodically. Water quality parameters should be checked regularly by authorized institutions.

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Chromosomal changes linked with the effect of high dose of aluminum on faba bean (*Vicia faba* L.) root tips

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Abstract. In this work, we cytogenetically described the effects of different relatively high doses of aluminum (5, 15 and 25 mM $AlCl_3$) on mitotic activity and DNA integrity of faba bean (Misr 3 cultivar). Under Al stress, mitotic index (MI) decreased but total chromosomal abnormalities increased significantly compared to that of control. In addition, the detected chromosomal abnormalities in each mitotic phase increased significantly in comparison to that of control. All the used concentrations of Al enhanced micronuclei formation; no correlation could be detected between the size or number of micronuclei/cell and the applied conditions. Metaphase was the most sensitive stage to Al stress compared to the other stages of mitosis; C-metaphase was the common abnormalities and it increased strongly when the exposure time was more than 6 h. Under the influence of Al stress for 24 h, the appearance C-metaphase in high frequency decreased the frequency of appearance of other forms of abnormalities during metaphase or ana-telophase. The previous cytological events created alteration either at or between the primer binding sites which could be detected by RAPD and ISSR techniques. Application of ten RAPD primers resulted in amplification of 59 fragments including 20 monomorphic, seven unique and 32 polymorphic bands with polymorphism average of 60.09%. ISSR primers amplified 75 DNA fragments including 18 monomorphic, eight unique and 45 polymorphic bands with polymorphism average of 72.90%. These data indicated that faba bean cultivar suffered from harmful effect of Al on its genome when the duration of Al treatment was more than 6 hr. ISSR was better than RAPD to study genome stability of faba bean under abiotic stress agent.

Keywords: aluminum stress, chromosomal changes, molecular techniques, cytogenetic effect, *Vicia faba*.

INTRODUCTION

In several environments, soils are contained with metals in different concentrations. They resulted in beneficial or toxic effects on biological sys-

tems that inhabit their environments; aluminum (Al) is one of those widespread metals. In nature, the highest amount of Al is present in un-soluble form as aluminum silicate. While soluble Al form is present in a small amount, it finds a way to get from soil, water and air to biological systems (May and Nordstrom 1991) leading to positive effects on cultivated plants (Foy 1983; Rout et al. 2001). On the other side, transfer of Al from soil to plants in relatively high dose resulted in catastrophic effects and they vary depending on plant species especially under low pH (5.5) of the soil (Rout et al. 2001).

In root tips, Aluminum intervenes with cell division, increases cell wall rigidity, increases the rigidity DNA double helix and reduces DNA replication (Foy 1992). Authors were also reported that Al (0.2–1.0 mM) inhibits cell division due to severe inhibition of DNA synthesis within 16 –24 h (Minocha et al. 1992). While mitotic indices decreased, anaphase chromosome aberrations increased when faba bean root tips of faba ben exposed to different doses of Al (0.01–10 mM) for 12 h (Yi et al. 2010). Under Al stress, reduction in mitotic activity in several plant species was reported (Rout et al. 2001; Silva 2012). Several studies indicated that Al toxicity increased micronuclei formation, chromosomal abnormalities and sister chromatid exchanges (Lima et al. 2007; Yi et al. 2010).

Genotoxic effects of stress agents were associated with chromosome aberration, micronucleus formation and chromosomal recombination (Achary and Panda 2010). Genotoxicity and DNA destroy can be evaluated by cytogenetical or molecular techniques. RAPD and ISSR used as reliable molecular tools to detect DNA variation, damage and mutational events in cells of animals, microorganisms and plants (Liu et al. 2005, 2009; Salem and Hassanein 2017; Hassanein et al. 2018). They depend on the fact that the primer is joined with complementary DNA sequence on opposite DNA strands of the studied genome. Under the influence of stress agent, primer binding sites vary under the influence of induced mutation and DNA damage (Gupta and Sarin 2009; Achary and panda 2010) leading to amplification of different DNA fragments expressing different polymorphism values. The value of polymorphism gives a clear indication of the range to which the genome is affected by the factor in question. Then, chromosomal destroy can be revealed by cytogenetical or molecular techniques.

Vicia faba belongs to Fabaceae family and used as human food and animal feed. In addition, bean and other legumes are used to improve the fertility of soil through nitrogen fixation. Aluminum has clear genotoxic and cytotoxic effects on cells of faba bean root tips (Yi et al. 2010). Consequently, faba bean root tips were used as a model plant to study the cytogenetic effects of *Dipterygium glaucum* extracts (Altwaty et al. 2016), herbicides and other materials on mitotic activity (El-Rokiek et al. 2015; Shafeek et al. 2016; Prabhu et al. 2017). Exposing the faba bean plant to pollutants in various concentrations is considered as a prominent test to determine the genotoxicity (Foltête et al. 2011; Prabhu et al. 2017). Prominent study reported that application of Al in low concentration induced adaptive response that led to genomic protection from genotoxic effects of Al or other materials (Achary and Panda 2010).

Under the influence of Al stress, inhibition the growth of plant roots was described as the most common symptoms exhibited by higher plants (Rout et al. 2001; Hassanein et al. 2020a). While several cytogenetical studies were explained the effects of Al toxicity on root tips, clear view still needs further studies using model plants such as faba bean. In addition, molecular phenomenon that underlies cytogenetical events are still not fully understood. In this work, we described the effects of application of relatively high doses of Al for different exposure periods on mitotic activity and DNA integrity of faba bean root tips using cytogenetical and molecular approaches.

MATERIAL AND METHODS

Plant materials

Seeds of *Vicia faba* cultivar (Misr 3) were obtained from the Agriculture Research and Seeds Center in Qena, Egypt. Seeds were germinated in distilled H_2O for two to three days to get roots with 2-3 cm long. The obtained roots were treated in solutions containing different concentrations of $AlCl_3$ (5, 15, 25 mM) for different periods (0, 6, 12 and 24 h).

Cytogenetical analysis procedure

Ten root tips of Al treated seedlings were cut and placed 12 h in Carnoy's fixation solution containing ethanol and acetic acid, glacial (3:1 ratio). The cut seedling root tips were kept in 70% ethanol under dark condition at 4°C, hydrolyzed in 1 N HCl (Darlington and La Cour 1976) and subjected to the Feulgen squash technique. Total mitotic, mitotic indices, the frequency of mitotic stages and mitotic chromosomal abnormalities were determined according to the detailed formulas in Hassanein et al. (2020b).

Table 1. The applied RAPD and ISSR primers.

Primer type	Primer Sequence (5'---------3')	Primer type	Primer Sequence (5'---------3')
RAPD		**ISSR**	
OPA-02	TGCCGAGCTG	ISSR1	ACACACACACACACACCTG
OPA-05	AGGGGTCTTG	ISSR2	CACACACACACACACAAAGCT
OPA-07	GAAACGGGTG	ISSR3	ACACACACACACACACAAG
OPA-17	GACCGCTTGT	ISSR4	GAGAGAGAGAGAGAGACTG
OPat-08	TCCTCGTGGG	ISSR5	GAGAGAGAGAGAGAGACTC
OPaw-10	GGTGTTTGCC	ISSR7	CTCTCTCTCTCTA (CT)6A
OPD-1	ACCGCGAAGG	ISSR8	TCTTCTTCTTCTG
OPD-18	GAGAGCCAAC	ISSR9	TGTTGTTGTGC
OPJ-15	TGTAGCAGGG	ISSR10	GTGGTGGTGGC
OPP-13	GGAGTGCCTC		

Genomic DNA extraction

Roots of 2-3 seedlings were treated with 5 mM Al for different periods (0, 6, 12 and 24 h) and subjected for genome analysis to investigate how the genome of faba bean was influenced by Al toxicity. Genomic DNA extraction was extracted from root tips and analyzed using RAPD and ISSR techniques according to Porebski et al. (1997).

PCR conditions

A total of ten RAPD and nine ISSR primers (Table 1) were used to detect induced genetic variation in faba bean roots under the influence of 5 mM Al for different periods (6, 12 and 24 hr). Genomic DNA amplification was fulfilled in a DNA Thermal Cycler (Biometra TPersonal Combi, Biometra GmbH, Germany). Application of RAPD and ISSR primers were carried out in a 25 µl PCR mixture solution containing 12.5 µl of Go Taq® Green Master Mix (Promega, Madison, USA), 3 µl of primer 10 pmol, 6.5 µl of free nuclease water and 3 µl of 100 ng genomic DNA templates. Then, PCR run for DNA amplification was started using initial denaturation cycle at 94°C for 5 min. Then, 40 cycles were carried out using denaturation (94°C for 45 sec), annealing step (optimized for each primer), and elongation (72°C for 1 min) steps. Extension step was used to finalize the amplification process at 72°C for 7 min.

Reproducibility

Reproducibility was taken in consideration to minimize personal errors. In this concern, each primer was used three times under the same PCR conditions.

Detection of PCR products

The obtained amplification products of each PCR run were electrophoresed in a 1.5% or 2% agarose gel containing 0.5 µg/ml ethidium bromide in 1X TBE. Run was carried out in run buffer at 70 volts. Then, the amplified PCR products were visualized, photographed and analyzed.

Data Analysis

The experimental data were statistically analyzed by ANOVA. Data were compared using the least significant difference (LSD) test at 5% (*) and 1% (**) levels (Snedecor and Cochran 1980). Also, dendrograms were generated for cluster analysis according to Legendre and Legendre (1983) using the Community Analysis Package Software Program (CAP) Version 4.0 (Richard and Peter 2007).

RESULTS

Under the influence of all $AlCl_3$ treatments, the decrease in MI and increase of total abnormalities were found to be statistically highly significant compared to that of control (Table 2). Irrespective the concentration of Al, the value of MI was gradually decreased as the exposure time increased. Roots treated with the highest concentration of $AlCl_3$ (25 mM) for 24 hr showed the highest inhibition of cell division and it was associated with the highest total abnormalities. In addition, the increase in interphase with increase of the concentrations of $AlCl_3$ and exposure time was appeared at all Al treatments.

Table 2. Mitotic index (MI), % of total abnormalities, % of interphase and % of mitotic phases (prophase, metaphase and ana-telophase), include normal (Total) and abnormal (Abn) mitotic phases recorded for *Vicia faba* (Misr 3) root tips under the influence of different concentrations of $AlCl_3$ (5, 15 and 25 mM) and exposure times (6, 12 and 24 hr).

Treatment		Total mitosis	Mitotic Index (MI)	% Total abnormal	% Interphase		% Prophase		% Metaphase		% Ana-telophase	
Exposure time	$AlCl_3$ conc.				Total	Abn.	Total	Abn.	Total	Abn.	Total	Abn.
6hr.	0 mM	355	7.10±0.36	4.09±0.42	92.9	0.4	37.6	0.4	24.7	1.4	42.2	-
	5 mM	121	2.40±0.10**	83.93±6.41**	97.5	2.33	70.5	65.8	10.6	10.6	16.5	10.5
	15 mM	99	1.95±0.15**	76.67±6.65**	98.0	1.37	66.6	62.8	7.7	7.7	25.6	7.7
	25 mM	126	2.50±0.10**	65.15±11.05**	97.4	2.6	36.2	25.8	15.5	13.8	44.8	22.4
12hr.	5 mM	79	1.60±0.10**	92.60±1.30**	98.4	0.79	51.7	48.2	39.3	39.2	8.9	5.4
	15 mM	64	1.26±0.05**	89.30±10.70**	98.7	2.5	56.5	43.4	30.4	30.4	13.04	13.1
	25 mM	47	0.93±0.15**	95.70±0.10**	99.0	1.26	29.8	29.7	61.7	59.5	8.5	6.4
24hr.	5 mM	51	1.00±0.10**	88.13±8.15**	98.9	0.71	61.7	55.3	38.2	38.3	-	-
	15 mM	86	1.70±0.00**	80.20±9.30**	98.2	0.76	76	50	26.0	26.0	8.0	2.0
	25 mM	37	0.75±0.05**	97.90±2.10**	99.2	1.11	30.6	27.8	69.4	69.4	-	-

**. The mean difference is significant at the 0.05 level.

Table 3. Types and frequency of chromosomal abnormalities [C-metaphase (C-m), sticky (Stick), star, break, disturbed (Dist), and diagonal (Diag), bridge, free and C-anaphase (C-ana)] recorded for *Vicia faba* (Misr 3) root tips under the influence of different concentrations of $AlCl_3$ and exposure times (6, 12, and 24 hr).

Treatment		Interphase abnormalities		Metaphase abnormalities					Ana-telophase abnormalities							
Exposure time	$AlCl_3$ conc.	Micro	Bi nuclei	C-m	Stick	Star	Break	Dist	Stick	Bridg	Dist	Diag	free	break	Star	C-ana
6hr.	0 mM	-	-	-	1.3	-	-	-	-	-	-	-	-	-	-	-
	5 mM	1.76	0.37	4.7	-	2.4	2.4	1.2	1.2	2.4	3.5	1.3	2.4	1.2	-	-
	15 mM	0.79	0.28	2.6	-	1.3	-	3.8	-	-	1.3	-	-	1.3	2.6	1.3
	25 mM	0.30	0.13	3.4	-	1.7	3.4	5.2	6.9	-	10.3	-	1.7	3.4	-	-
12hr.	5 mM	1.09	-	39.3	-	-	-	-	-	-	-	-	-	-	1.8	3.6
	15 mM	2.53	-	26.1	-	-	-	4.3	8.7	4.3	-	4.2	-	-	-	-
	25 mM	0.31	0.04	57.4	-	-	2.1	-	-	-	-	-	-	-	2.1	-
24hr.	5 mM	2.39	0.41	38.3	-	-	-	-	-	-	-	-	-	-	-	-
	15 mM	1.22	0.45	18	-	-	-	-	-	-	-	-	-	-	2	-
	25 mM	1.05	0.06	66.7	-	2.8	-	-	-	-	-	-	-	-	-	-

The relative frequencies of different mitotic phases were affected by $AlCl_3$ treatments (Table 2). Variations in these frequencies appeared to be dependent on exposure time and concentrations of the applied $AlCl_3$ concentrations. Prophase frequency and prophase abnormalities (Fig. 1:4) usually increased as the concentration of $AlCl_3$ increased and the exposure time prolonged up to 15 mM $AlCl_3$. Prophase minimum values of 36.2, 29.8, and 30.6% were observed when root tips were subjected to 25 mM $AlCl_3$ for 6, 12 and 24 h, respectively. Metaphase frequency mostly increased as the concentration of $AlCl_3$ increased and exposure time was prolonged for more than 6 h; it was associated with increase in metaphase abnormalities. Maximum value of metaphase frequency (69.4%) was detected when plant root tips were subjected to 25 mM $AlCl_3$ for 24 hr. When plant root tips were subjected to $AlCl_3$ toxicity, the registered values of ana-telophase frequency were generally lower than that of control. Under the influence of 5 or 25 mM $AlCl_3$ for 24 h a complete inhibition in ana-telophase stage

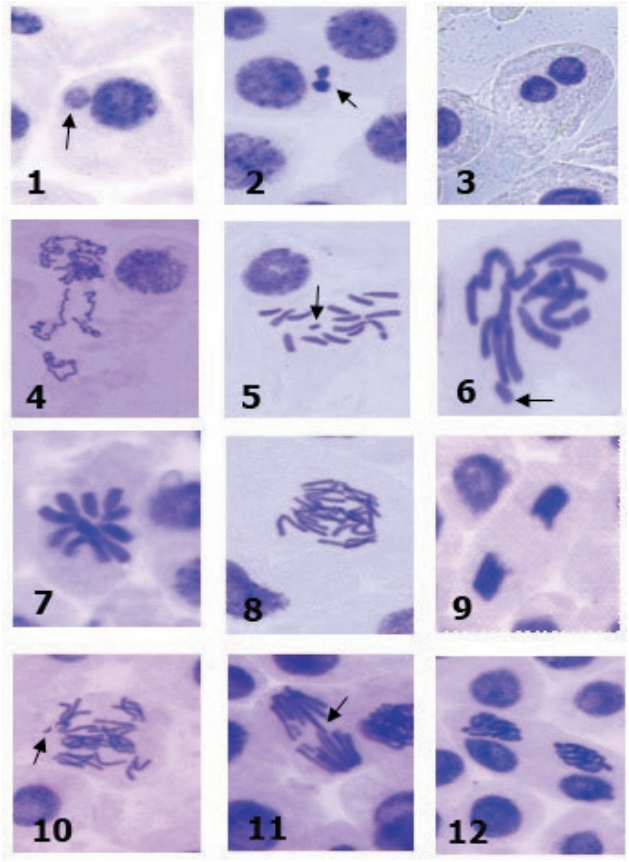

Figure 1. Chromosomal aberrations of Misr 3 cultivar root tips under the influence of $AlCl_3$ for different periods (6, 12, and 24 hr); (1, 2) micronuclei formation during interphase stage (arrows), (3) binucleated interphase, (4) irregular prophase, (5) C-metaphase with breaks (arrow), (6) disturbed metaphase, (7) star metaphase, (8) disturbed anaphase, (9) sticky anaphase, (10) breaks in anaphase (arrow), (11) bridge anaphase (arrow) and (12) star shape anaphase.

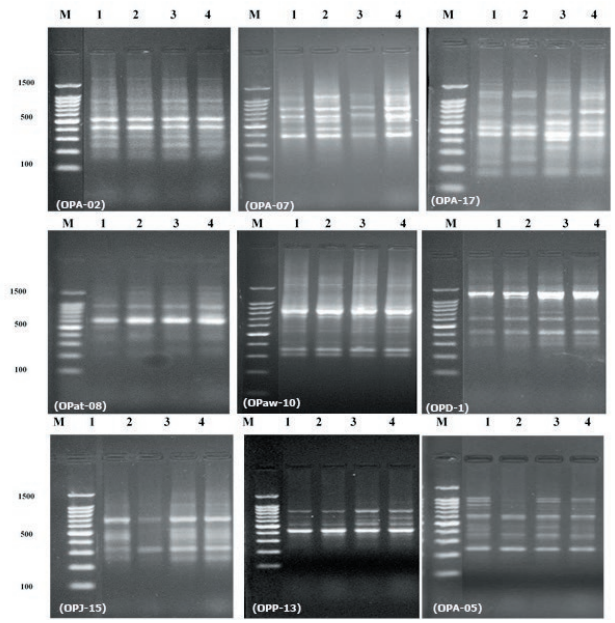

Figure 2. RAPD profile generated by 9 primers using roots of different *Vicia faba* Misr 3 cultivar subjected for different periods in 5 mM $AlCl_3$. Lanes1, 2, 3 and 4: 0.0, 6, 12 and 24 hr, respectively. M: DNA ladder.

was detected. The detected abnormalities in each mitotic phase were higher than that of control.

All the used concentrations of $AlCl_3$ enhanced micronuclei formation in the root tips (Table 3 and Fig. 1). The maximum value of micronuclei frequency (2.53%) was detected when root tips were subjected to 15 mM for 12 hr. Under all the applied conditions, one (Fig. 1: 1), or two (Fig. 1: 2) micronuclei/cell were detected. There was no correlation between the number of micronuclei/ cell and the applied concentration of $AlCl_3$ or the exposure time. Also, binucleated cells were detected under the applied conditions (Fig. 1: 3).

The type and frequency of chromosomal abnormalities that resulted from $AlCl_3$ treatments on faba bean (Misr 3) roots were included in Table 3 and illustrated in Fig.1. Metaphase was the most sensitive stage to Al stress compared to the other stages of mitosis, where the abnormalities were detected at all treatments (Table 3). C-metaphase (Fig. 1: 5) was the common abnormalities and their appearance was independent on Al concentration but it increased strongly when the exposure time was more than 6 h. In addition, during this stage, abnormalities including star shape (Fig. 1: 7), chromosomes breaks (Fig. 1: 5, 6) and disturbed configurations (Fig. 1: 6) were detected. Under the influence of the used high doses of Al for the longest period of exposure (24 h), MI was drastically lowered but total abnormalities were increased and it was associated with drastic increase in C-metaphase. The concentration of most of the chromosomal abnormalities of the metaphase stage in one form of alteration (C-metaphase) made the appearance of other forms of abnormalities rare during metaphase or telo-anaphase.

Ana-telophase chromosomal abnormalities including chromosomal bridges (Fig. 1: 11), chromosomal breaks (Fig. 1: 10), disturbed anaphase (Fig. 1: 8) and sticky anaphase (Fig. 1: 9) were detected. When plant root tips were subjected to the relatively high concentration of $AlCl_3$ (15 mM) for relatively long period (24 h), star shape chromosome was detected (Fig. 1: 12) but other types of abnormalities were not registered during anatelophase stage. The same results were obtained when plant root tips were treated with 25 mM $AlCl_3$ for 12 hr. Under different Al treatments, star chromosome was

Table 4. Ten RAPD primers, their sequences, annealing temperature, size of amplified fragments (bp), total number of amplified fragments, number of polymorphic bands and unique bands identified per primer used to access genome stability of *Vicia faba* Misr 3 cultivar under the influence of Al stress.

Primer	Sequence (5'→3')	Annealing temperature (°C)	Polymorphic bands	Monomorphic bands	Unique bands	Total bands	Size range (bp)	Polymorphism (%)
OPA-02	TGCCGAGCTG	32	4	2	1	7	141-753	71.43
OPA-05	AGGGGTCTTG	30	4	3	0	7	225-1102	57.14
OPA-07	GAAACGGGTG	30	3	2	0	5	497-1428	60.00
OPA-17	GACCGCTTGT	30	8	1	4	13	162-1811	92.31
OPat-08	TCCTCGTGGG	32	1	1	0	2	218-958	50.00
OPaw-10	GGTGTTTGCC	30	4	1	0	5	313-912	80.00
OPD-1	ACCGCGAAGG	32	2	3	1	6	236-1364	50.00
OPD-18	GAGAGCCAAC	30	0	4	0	4	292-1278	00.00
OPJ-15	TGTAGCAGGG	30	4	1	0	5	229-737	80.00
OPP-13	GGAGTGCCTC	32	2	2	1	5	345-876	60.00
Total			32	20	7	59		60.09

Table 5. Sequences and annealing temperature of nine ISSR primers were used to access genetic disorder of the root tip of *Vicia faba* (Misr 3 cultivar) as influenced by Al concentration and exposure time. Size of amplified fragments (bp), total number of amplified fragments, number of polymorphic bands, unique bands and specific bands identified per primer were included.

primer	Sequence (5'→3')	Annealing temperature (°C)	Polymorphic bands	Monomorphic bands	Unique bands	Total bands	Size range (bp)	Polymorphism (%)
ISSR1	ACACACACACACACACCTG	56	7	3	0	10	175-1287	70.00
ISSR2	CACACACACACACACAAAGCT	60	5	1	0	6	239-703	83.33
ISSR3	ACACACACACACACACAAG	58	6	1	2	9	206-870	88.89
ISSR4	GAGAGAGAGAGAGAGACTG	50	4	2	2	8	167-985	75.00
ISSR5	GAGAGAGAGAGAGAGACTC	50	2	6	2	10	188-565	40.00
ISSR7	CTCTCTCTCTCTA (CT)6A	38	6	1	1	8	804-1376	87.50
ISSR8	TCTTCTTCTTCTG	36	5	2	0	7	295-1489	71.42
ISSR9	TGTTGTTGTGC	32	7	0	0	7	169-828	100
ISSR10	GTGGTGGTGGC	38	3	6	1	10	457-1557	40.00
Total			45	18	8	75		72.90

the common type of chromosomal abnormalities during ana-telophase stage.

Root tips of Misr 3 cultivar were subjected to molecular analysis to reveal genome variation which in consistent with chromosomal abnormalities under Al toxicity (Table 4). In this concern, ten RAPD primers were used (Fig. 2). Misr 3 cultivar showed 32 polymorphic (54.24 %), 20 monomorphic bands (33.89 %) and seven unique bands (11.86 %) out of 59 fragments. The number of bands ranged from two using OPat-08 primer to 13 bands using OPA-17 primer. Percentage of polymorphism ranged from 0 % when OPD-18 was used to 92.31 % using OPA-17 with an average of 60.09 %. Primer OPA-17 gave the highest number of polymorphic RAPD markers. The average number of bands per polymorphic primers was 5.9 and the average number of polymorphic bands per polymorphic primers was 3.9. The highest number of bands was detected when OPA-17 primer was used. The size of the obtained fragments using all the RAPD primers ranged from 141 to 1811 bp.

Nine ISSR primers were used for amplification of genomic DNA of *V. faba* Misr 3 cultivar (Table 5 & Fig. 3). Under the application of these nine primers 75 amplified fragments were detected. Forty-five of them were polymorphic (60 %), 18 were monomorphic bands (24 %) and eight were unique bands (10.67 %). The number of bands per primers ranged from six using ISSR2 primer to ten bands using ISSR1, ISSR5 and ISSR10 primers. The size of the obtained fragments using all ISSR primers varied between 167-1557 bp. Primers ISSR1 or ISSR9

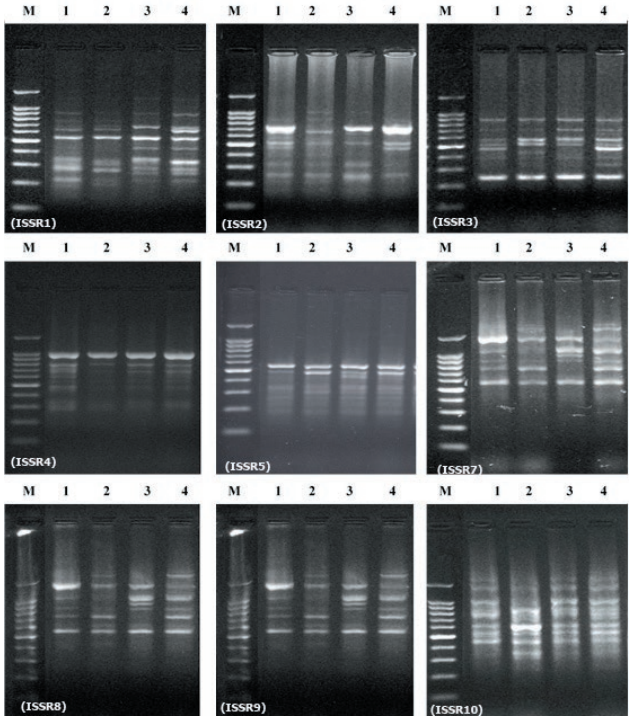

Figure 3. ISSR-PCR profiles generated by 9 primer using roots of different *V. faba* Misr 3 cultivar subjected for different periods in 5 mM $AlCl_3$. Lanes1, 2, 3 and 4 (0.0, 6, 12 and 24hr), respectively. M: DNA ladder.

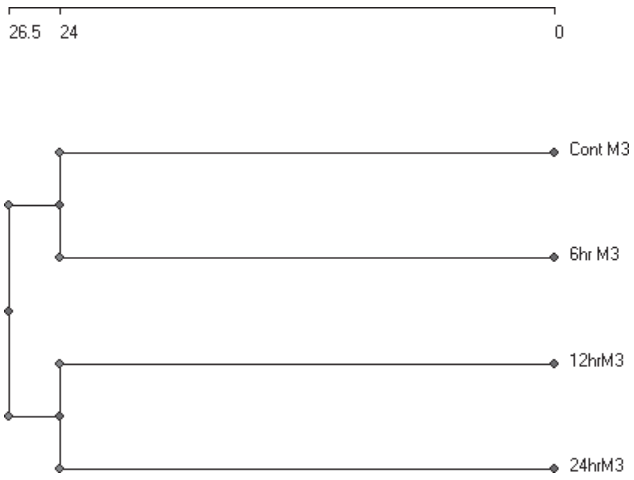

Figure 4. UPGMA based cluster tree of *Vicia faba* (Misr 3) cultivar exposed to 5 mM $AlCl_3$ for (0.0, 6, 12 and 24hr) based on (9 ISSR primers).

gave the highest number of polymorphic ISSR fragments. The average number of bands per polymorphic primers was 8.33 and the average number of polymorphic bands per polymorphic primers was 5.9. Percentage of polymorphism ranged from 40 % when ISSR5 and ISSR10 were used to 100 % using ISSR9 with an average of 72.90 %.

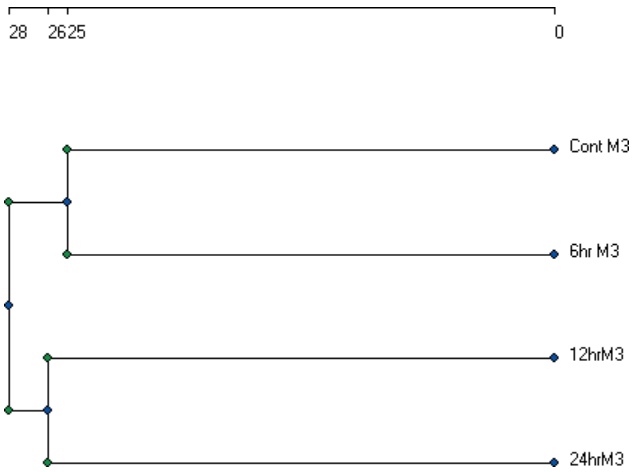

Figure 5. UPGMA based cluster tree of *Vicia faba* (Misr 3) cultivar exposed to 5 mM $AlCl_3$ for (0.0, 6, 12 and 24hr) based on (10 RAPD + 9 ISSR primers).

When results of the application of ISSR were compared with others of RAPD primers, valuable variations were detected. Total bands per nine primers of ISSR (75) were higher than that of ten RAPD (59) primers. In addition, the average polymorphism obtained from the application of ISSR primers (72.90 %) was higher than the average polymorphism obtained from applying of RAPD primers (60.09 %).

Cluster tree based upon UPGMA analysis of ten RAPD and nine ISSR primers of *V. faba* Misr3 cultivar resulted in two main clusters (Fig. 4 & 5). The first cluster included control and 6 h treated roots with $AlCl_3$. The second cluster included 12 and 24 h treated roots with $AlCl_3$. The obtained dendrogram as well as cytogenetical and polymorphism data showed that Misr 3 cultivar suffered from harmful effect of Al on the nuclear genome, especially when the duration of exposure time was more than 6 hr.

DISCUSSION

In this work, seedlings of faba bean were subjected to genotoxicity of high dose Al (5- 25 mM $AlCl_3$), the obtained nuclear genome variation was registered using cytogenetical and molecular approaches. Cytogenetical effects of Al on faba bean (Yi et al. 2010; Hassanein et al. 2020b) and other plant species (Li et al. 2015; Domingues et al. 2012; Jaskowiak et al. 2018) were studied. In this work, irrespective the concentration of Al,

the value of MI was gradually decreased as the exposure time increased as was also reported by other authors (Yi et al. 2010; Altwaty et al. 2016; Hassanein et al. 2020b).

Variations in frequencies of different mitotic phases were registered and indicated that Al induced cell cycle alterations in faba bean as was reported by Yi et al. (2010). Consequently, prophase frequency increased as the concentration of $AlCl_3$ increased and exposure time prolonged up to 15 mM $AlCl_3$. Also, metaphase frequency mostly increased as the concentration of $AlCl_3$ increased except plant roots subjected Al stress for 6 hr. Increase of prophase and metaphase frequencies associated with increase in their abnormalities. In addition, increase in prophase frequency associated with decrease in ana-telophase frequency under Al treatments and it showed complete inhibition when Al stresses were applied for 24 h.

Micronuclei were detected in the root tips exposed to different concentrations of $AlCl_3$ for different periods and ranged from small to large size. There was no correlation between the size or number of micronuclei/cell and the applied conditions. Binucleated cells are formed in Al stressed Misr 3 cultivar as the result of disturbed cytokinesis (Alberts et al. 2002) and cell plate formation (Gisselsson et al. 2001). Micronuclei frequency is a good indicator of the cytogenetic effects of tested chemicals such as $AlCl_3$ in faba bean and other plant species (Kanaya et al. 1994; Gecheff 1996; Grant and Owens 1998; Kundu and Ray 2017). Consequently, micronucleus assay was considered as the efficient, simplest and most effective tool to measure of chromosomal DNA damages and analyze the mutagenic effect of different substances (Auerbach 1962; El-Azab et al. 2018; Kursheed et al. 2018).

Under the used high doses of $AlCl_3$, metaphase was the most sensitive stage compared to the other stages of mitosis, where the abnormalities were detected at all treatments. In addition, C-metaphase was the common abnormalities, and their appearance was independent on Al concentration, but it increased strongly when the exposure time was more than 6 hr. In addition, metaphase abnormalities including chromosomal stickiness, star shape chromosomes, chromosomal breaks and disturbed configurations were detected. During metaphase, failure of broken chromosome to recombine correctly due to the stickiness of chromosomes and their inability to arrive to the poles led to the appearance of chromosomal breaks under Al or other stress agent (Agarwal and Ansari 2001). These abnormalities were also detected by other authors in faba bean (Yi et al. 2010; Hassanein et al. 2020b) and other plant species (De Campos and Viccini 2003; Mohanty et al. 2004).

Concentration abnormalities in one form (C-metaphase) during metaphase made the appearance of other forms of abnormalities rare during metaphase or telo-anaphase, especially under long exposure time (24 h). In addition, ana-telophase chromosomal abnormalities including chromosomal bridges, chromosomal breaks, disturbed anaphase, diagonal and star chromosomes, and C-anaphase were detected under Al treatments. The registered stickiness in faba bean and other plant species gave an indication about the direct destructive effect of a toxic agent on chromosomes (Renjana et al. 2013) or spindle disturbance (Gaulden 1987). Lagging chromosomes and chromosomal bridges were appeared which may be due to abnormal spindle fiber formation (Badr et al. 2013). Also, Rieger et al. (1991) believed that the inhibition of cytoskeletal proteins leads to the formation of lagging chromosomes.

Data of our study indicated that Al treatments in high dose induced chromosomal abnormalities and micronuclei formation. These two phenomenon may result from inhibition of DNA synthesis (Minocha et al. 1992), alteration of chemical or electrostatic properties of DNA (Unrau and Laster 1952), elimination of genetic material (Fernandes et al. 2007), induction of DNA fragmentation (Jaskowiak et al. 2018), formation of chromosomal bridges and chromosomal breaks (Ignacimuthu and Babu 1989), miss-repair of the broken DNAs or fused of telomeres (Souguir et al. 2018), stickiness of chromosomes (Badr et al. 2014). In addition to point mutation, the previous events created alteration either at or between the primer binding sites, which could be detected by RAPD and ISSR techniques (Liu et al. 2007; Gupta and Sarin 2009). During PCR program, binding between primers and complemented loci resulted amplification of DNA fragments with molecular weight of 100 to 2000 bp (Ng and Tan 2015). The amplified DNA fragments were dependent on the extent of chromosomal changes under the toxic effect of Al due to the previous events. These events created or abolished some primer binding sites leading to polymorphism. This means that the detected DNA polymorphism may be due to mismatching at the primer site, appearance of a new primer site and/or change the distance between two opposite primers. Using RAPD primers, Misr 3 cultivar showed 32 polymorphic out of 59 fragments primers (54.24 %), 20 monomorphic bands (33.89 %) and seven unique bands (11.86 %). Percentage of polymorphism ranged from 0 % when OPD-18 was used to 92.31 % using OPA-17 with an average of 60.09 %. Under the application of ISSR primers, polymorphism average was 72.90 %. The average polymorphism obtained from the application of ISSR primers

(72.90 %) was higher than that of RAPD primers (60.09 %). Then, ISSR is an ideal molecular marker to study natural or induced genetic variation of faba bean cultivars as well as other plants (Abdel-Razzak et al. 2012; Alghamdi et al. 2012; Wang et al. 2012; Salem and Hassanein 2017; Hassanein et al. 2018).

Cluster tree based on RAPD and ISSR primers indicated that Misr 3 cultivar suffered from harmful effect of Al on its genome when plant root tips were exposed to Al for more than 6 h where great cytogenetical events were happened and resulted in variation in primer binding sites leading to high polymorphism. In faba bean, Yi et al. (2010) reported that significant increase in frequencies of micronuclei formation and chromosome aberrations was induced when root tips were exposed to 0.01–10 mM Al for 12 hr. Consequently, Molecular markers, e.g. ISSR, could be used to confirm the cytogenetical data and supplemented us with valuable information about metal genotoxicity such as Al, especially when they were used in relatively high concentrations.

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