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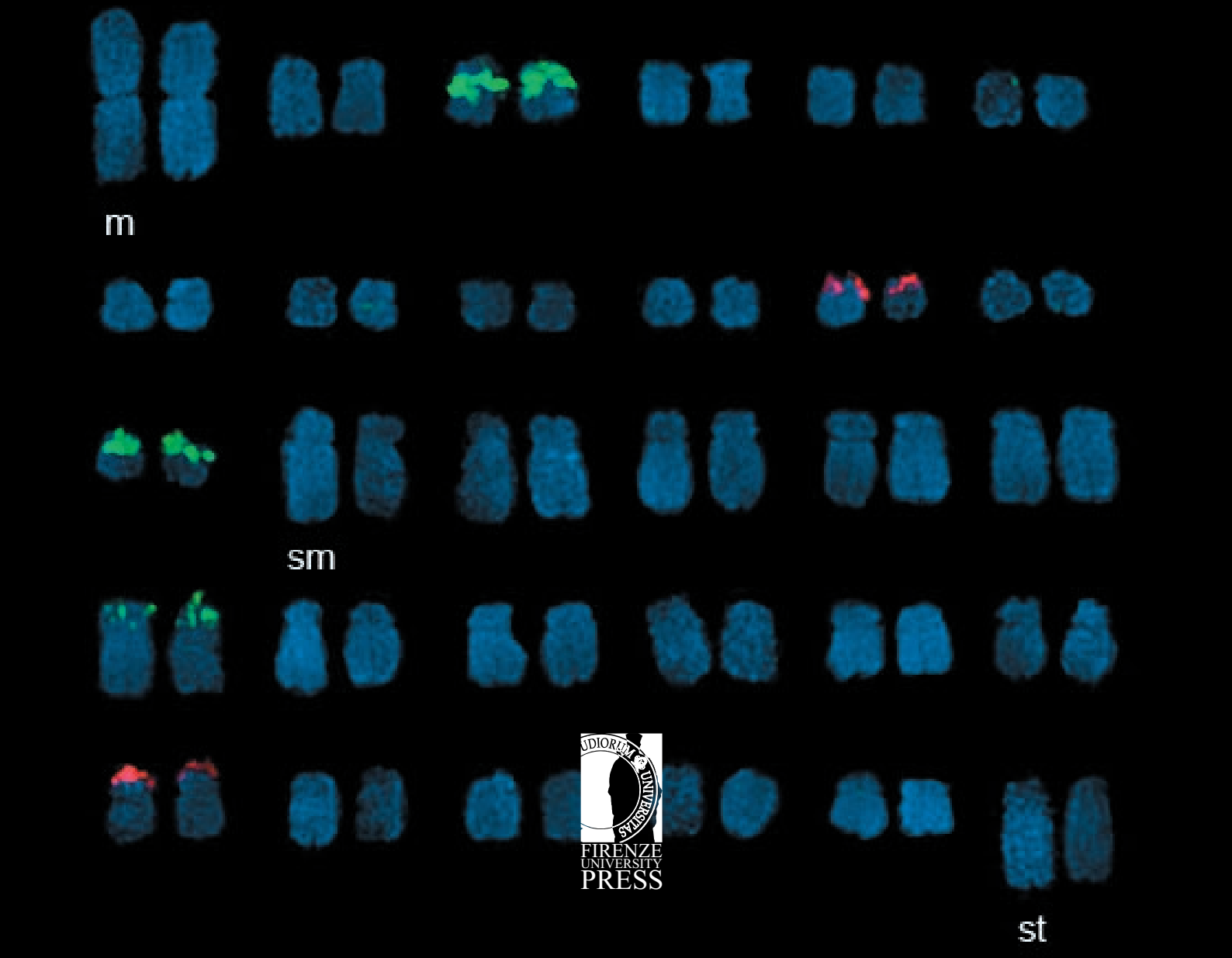

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Species delimitation in the genus *Cousinia* Cass. (Family Asteraceae), sections *Cynaroideae* Bunge and *Platyacanthae* Rech. f.: morphometry and molecular analysis

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Abstract. The genus *Cousinia* of the tribe Cardueae with about 700 species is one of the most diverse genera in Central and southwest Asia. The section *Cynaroides* with 89 species is the largest section of the genus. Due to the controversy in the number of *Cousinia* species and their delineation, the first step in studying the genus is to identify and delimit presumed species. Species delimitation is usually difficult in the species with overlaps in their morphological features. Therefore, we used a combination of morphological and molecular markers (ISSRs) to carry out delimitation in 204 taxa of 68 *Cousinia* species whitin the *Cynaroideae* and *Platyacanthae* sections. The species delineation based on morphometry and ISSR data were done by UPGMA clustering. The samples of each species were placed close to each other and formed a single sub-cluster, separated from the other studied *Cousinia* species. In the present study the studied *Cousinia* species within *Cynaroideae* and *Platyacanthae* sections could be delimited from each other based on ISSR and morphological data. Therefore, using ISSR and morphological data can be useful in identifying and delineating crucial species. The Mantel test performed between morphological distance and Nei genetic distance produced non-significant correlation. This result also supports distance analyses of the trees and reveals that the two dendrograms are not correlated. Some possible reasons for this incongruence are proposed: the high number of taxa in the genus *Cousinia,* morphological traits homoplasious, convergent evolution and incomplete lineage sorting.

Keywords: *Cousinia, Cynaroideae,* ISSR, morphometry.

INTRODUCTION

The genus *Cousinia* Cass. of the tribe Cardueae (Family Asteraceae) with about 700 species is one of the most diverse genera in Central and southwest (SW) Asia After *Senecio* L. (c. 1500 species) and *Vernonia* Schreb. (c. 1000 species) (Tscherneva 1962; Rechinger 1972, 1979; Frodin 2004; Attar and

Ghahreman 2006; Susanna and Garcia-Jacas 2006; Attar and Djavadi 2010; Mehregan and Assadi 2016; Minaeifar *et al.* 2016; Rastegar *et al.* 2017, 2018). Due to the extensive morphological variability in the genus, *Cousinia* taxonomy is complicated and controversial (Mabberley 1990; Haffner 2000; Susanna *et al.* 2003). The genus *Cousinia* contains more than 400 species in SW Asia, with the highest number of species in the Flora Iranica area, out of which 379 are endemic. These species are distributed in mountainous regions of Iran, Afghanistan and Turkmenistan (Rechinger 1986; Knapp 1987). Although the exact number of *Cousinia* species in Iran is still unknown, about 270 species have been reported till now (Assadi 2009; Attar and Djavadi 2010). Out of these, nearly 200 endemic *Cousinia* species occur in Iran (Djavadi *et al.* 2007; Zare *et al.* 2013). The *Cousinia* species are distributed in 70 sections (Rechinger 1986). The section *Cynaroideae* Bunge with 89 species is the largest section of the genus and contains Irano-Turkestanian elements (Tscherneva 1962; Rechinger 1972, 1979; Huber- Morath 1975; Attar and Djavadi 2010; Rastegar *et al.* 2017, 2018). This sect. includes those species consisting of decurrent leaves and appendiculate bracts (Tscherneva 1962; Rechinger 1972, 1979; Huber- Morath 1975). Iran with 77 taxa, of which 66 are endemic, seems to be the centre of diversity of the section *Cynaroideae* (Attar and Ghahreman 2006). The section *Platyacanthae* Rech. f. has 6 species in Flora Iranica of which 5 species are endemic in Iran (Rechinger 1972).

Due to the controversy in the number of *Cousinia* species and their delineation, the first step in studying the genus is to identify and delimit presumed species. Species delimitation is usually difficult in the species with overlaps in their morphological features (Wiens 2007). In such cases, combined morphological and molecular data have been used to delimit these taxonomic identities (Duminil and Di Michele 2009; Minaeifar *et al.* 2016; Hassanpour *et al.* 2018; Eftekharian *et al.* 2018).

Different molecular markers have been used in plant taxonomy and phylogeny, but some of them such as inter-simple sequence repeats (ISSRs) seems to be very efficient in delineating species, varieties, ecotype and even genotypes of a single species (See for example, Sheidai *et al.* 2012, 2013; Safaei *et al.* 2016; Eftekharian *et al.* 2018). Therefore, we used a combination of morphological and molecular markers (ISSRs) to carry out *Cousinia* species delimitation in sections *Cynaroideae* and *Platyacanthae.*

MATERIALS AND METHODS

Plant material

The data investigated and discussed in the present study are based on 204 samples of 68 species in of the sections *Cynaroideae* and *Platyacanthae*. Sixty-three species (189 specimens) of *Cynaroideae* and five species (15 specimens) of *Platyacanthae* were selected. The plant samples were collected from Iran (Table 1). The vouch-

Table 1. Investigated *Cousinia* species and their voucher information.

No	Taxa	Section	Locality	Voucher no.
1	*C. keredjensis* Bornm. & Gauba	*Cynaroides* Bunge	Tehran	21807(TUH)
2	*C. elwendensis* Bornm.	*Cynaroides* Bunge	Hamadan-Alvand Mountains	20566(TUH)
3	*C. grandis* C. A. Mey.	*Cynaroides* Bunge	Azarbaijan	21343(TUH)
4	*C. disfulensis* Bornm.	*Cynaroides* Bunge	Lorestan- Khorram Abad	27589(TUH)
5	*C. bornmulleri* C. Winkl.	*Cynaroides* Bunge	Esfahan	22532(TUH)
6	*C. behboudiana* Rech. f. & Esfand.	*Cynaroides* Bunge	Ghazvin	27629(TUH)
7	*C. inflata* Boiss. & Hausskn.	*Cynaroides* Bunge	Kurdestan	39552(TUH)
8	*C. eriocephala* Boiss. & Hausskn.	*Cynaroides* Bunge	Azarbaijan	22442(TUH)
9	*C. calocephala* Jaub. & Spach	*Cynaroides* Bunge	Azarbaijan-Mianeh	46276(TUH)
10	*C. farsistanica* Bornm.	*Cynaroides* Bunge	Kerman	28636(TUH)
11	*C. jaccobsii* Rech. f.	*Cynaroides* Bunge	Ilam	22370(TUH)
12	*C. denaensis* Attar & Djavadi	*Cynaroides* Bunge	Boyer-Ahmad	22495(TUH)
13	*C. concinna* Boiss. & Hausskn.	*Cynaroides* Bunge	Kurdestan	20562(TUH)
14	*C. grantii* Rech. f.	*Cynaroides* Bunge	Azarbaijan	22490(TUH)
15	*C. bobeckii* Rech. f.	*Cynaroides* Bunge	Ardabil	46221(TUH)
16	*C. barbeyi* C. Winkl.	*Cynaroides* Bunge	Boyer-Ahmad	22494(TUH)
17	*C. kirrindica* Bornm. & Rech. f.	*Cynaroides* Bunge	Ilam	19711(TUH)

No	Taxa	Section	Locality	Voucher no.
18	*C. khorramabadensis* Bornm.	*Cynaroides* Bunge	Lorestan	21851(TUH)
19	*C. lactiflora* Rech. f.	*Cynaroides* Bunge	Lorestan	46299(TUH)
20	*C. phyllocephala* Bornm. & Gauba	*Cynaroides* Bunge	Lorestan- Khorram Abad	46292(TUH)
21	*C. lurorum* Bornm.	*Cynaroides* Bunge	Kermanshah- Mahidasht	20568(TUH)
22	*C. verbascifolia* Bunge	*Cynaroides* Bunge	Khorasan-Mashhad	43013(TUH)
23	*C. monocephala* Bunge	*Cynaroides* Bunge	Khorasan- Ghouchan	21931(TUH)
24	*C. shebliensis* Ghahreman	*Cynaroides* Bunge	Azarbaijan- Tabriz	20580(TUH)
25	*C. millefontana* Rech. f.	*Cynaroides* Bunge	Kurdestan-Marivan	20227(TUH)
26	*C. sanandajensis* Rech. f.	*Cynaroides* Bunge	Hamadan	46287(TUH)
27	*C. zardkuhensis* Attar & Ghahreman	*Cynaroides* Bunge	Chahar Mahal& Bakhtiari	21887(TUH)
28	*C. pergamacea* Boiss. & Hausskn.	*Cynaroides* Bunge	Kurdestan	22571(TUH)
29	*C. macrocephala* C. A. Mey.	*Cynaroides* Bunge	Ardebil- Meshkin shahr	42925(TUH)
30	*C. onopordioides* Ledeb.	*Cynaroides* Bunge	Khorasan: Kashmar	28685(TUH)
31	*C. aligudarzensis* Attar & Ghahreman	*Cynaroides* Bunge	Lorestan-Aligudarz	27613(TUH)
32	*C. dalahuensis* Attar & Ghahreman	*Cynaroides* Bunge	Kermanshah- Mahidasht	19929(TUH)
33	*C. carolihenrici* Attar & Ghahreman	*Cynaroides* Bunge	Kurdestan	22455 (TUH)
34	*C. khansarica* Attar & Ghahreman	*Cynaroides* Bunge	Esfahan: Khansar	20037(TUH)
35	*C. lurestanica* Attar & Djavadi	*Cynaroides* Bunge	Lorestan	21824(TUH)
36	*C. parsana* Ghahreman	*Cynaroides* Bunge	Hamadan	20553(TUH)
37	*C. pasargadensis* Attar	*Cynaroides* Bunge	Fars: Dashte Arjan	36294(TUH)
38	*C. perspolitana* Attar & Ghahreman	*Cynaroides* Bunge	Fars: Abadeh	22509(TUH)
39	*C. silvanica* Attar	*Cynaroides* Bunge	W Azarbaijan: Urmie	24064(TUH)
40	*C. shulabadensis* Attar & Ghahreman	*Cynaroides* Bunge	Lorestan- Shul Abad	21874(TUH)
41	*C. algurdina* Rech. f.	*Cynaroides* Bunge	Azarbaijan- Tabriz	30533(TUH)
42	*C. mobayenii* Ghahreman & Attar	*Cynaroides* Bunge	Kermanshah- Eslamabad	20569(TUH)
43	*C. sabalanica* Attar	*Cynaroides* Bunge	Ardebil	22570(TUH)
44	*C. kurdistanica* Attar	*Cynaroides* Bunge	Kurdestan- Maryvan	3232(TUH)
45	*C. gaharensis* Attar & Djavadi	*Cynaroides* Bunge	Lorestan- Shulabad	38259(TUH)
46	*C. kermanshahensis* Attar	*Cynaroides* Bunge	Kermanshah: Eslam-Abad	19810(TUH)
47	*C. fursei* Rech. f.	*Cynaroides* Bunge	Kurdestan-Marivan	18314(TUH)
48	*C. chlorosphaera* Bornm.	*Cynaroides* Bunge	Chahar Mahal& Bakhtiari: Soreshjan	26244(TUH)
49	*C. cynaroides* C. A. Mey	*Cynaroides* Bunge	Ardebil	22581(TUH)
50	*C. gilliatii* Rech. f.	*Cynaroides* Bunge	Azarbaijan	21967(TUH)
51	*C. iranica* C. Winkl. & Strauss.	*Cynaroides* Bunge	Arak	21881(TUH)
52	*C. kotschyi* Boiss.	*Cynaroides* Bunge	Azarbaijan	46244(TUH)
53	*C. kopikaradaghensis* Rech. f.	*Cynaroides* Bunge	Kurdestan: Saqqez	(TUH)
54	*C. sagittata* C. Winkl. & Strauss.	*Cynaroides* Bunge	Arak	21822(TUH)
55	*C. nana* Attar	*Cynaroides* Bunge	Arak	14347(TUH)
56	*C. sahandica* Attar & Djavadi	*Cynaroides* Bunge	Azarbaijan	46272(TUH)
57	*C. lordeganensis* Mehregan	*Cynaroides* Bunge	Chahar Mahal& Bakhtiari	46301(TUH)
58	*C. hamadanensis* Rech. f.	*Cynaroides* Bunge	Hamadan- Malayer	46290(TUH)
59	*C. subinflata* Bornm.	*Cynaroides* Bunge	Kermanshah	(TUH)
60	*C. kornhuberi* Heimerl	*Cynaroides* Bunge	Hamadan	22372(TUH)
61	*C. sardashtensis* Rech. f.	*Cynaroides* Bunge	Chahar Mahal& Bakhtiari	20073(TUH)
62	*C. sefidiana* Rech. f.	*Cynaroides* Bunge	Lorestan	21861(TUH)
63	*C. platyacantha* Bunge	*Platyacanthae* Rech. f.	Khorasan	43212(TUH)
64	*C. freynii* Bornm.	*Platyacanthae* Rech. f.	Semnan- Shahrud	27675(TUH)
65	*C. reshingerorum* Bornm.	*Platyacanthae* Rech. f.	Khorasan-Torbate Jam	39729(TUH)
66	*C. bienerti* Bunge	*Platyacanthae* Rech. f.	Khorasan-Neyshabur	28682(TUH)
67	*C. trachyphyllaria* Bornm. & Rech. f.	*Platyacanthae* Rech. f.	Khorasan- Ghouchan	21932(TUH)
68	*C. ecbatanensis* Bornm.	*Cynaroides* Bunge	Hamadan	22371(TUH)

er specimens have been deposited in The Herbarium of Tehran University (TUH) (Table 1).

DNA extraction and PCR amplification

Total genomic DNA was extracted from leaf tissue using protocol of the CTAB-activated charcoal and Polyvenyl Pyrrolidone (PVP) method (Murray and Thompson 1980). Quality of extracted DNA was examined by running on 0.8% Agarose gels. Each 20 ml PCR mixture contained 10 ml of 2_ PCR buffer, 0.5 mM of each primer, 200 mM of each dNTP, 1 Unit of Taq DNA polymerase (Bioron, Ludwigschafen, Germany), and 1 ml of template genomic DNA at 20 ng mle1. The PCR amplification program was performed in a Techne thermocycler (Germany) with the following program: 5 min at 94 °C, followed by 45 cycles of 30 s at 94 °C, 30 s at 54.6 °C, and 2 min at 72 °C, with a final extension step of 10 min at 72 C. The amplification products were visualized by running on 2% agarose gel, followed by ethidium bromide staining. The fragments size was estimated by using a 100-bp molecular size ladder (Fermentas, Germany). The experiment was replicated 3 times and constant ISSR bands were used for further analyses. Ten ISSR primers, UBC 807, UBC 810, UBC 811, UBC 834, CAG(GA)7, (CA)7AC, (CA)7AT, (CA)7GT (GA)9A, and (GA)9T, commercialized by the University of British Columbia, were used (Godwin *et al.* 1997).

Morphological analysis

In total, 19 morphological characters (quantitative and qualitative) were studied (Table 2). Morphological characters were coded accordingly. Data were standardized (mean = 0, variance = 1) and used for multivariate analyses. UPGMA (Unweighted paired group using average), and Ward (Minimum spherical variance) clustering based on Euclidean distance and Gower distances as well as principal coordinate analysis (PCoA) and multidimensional scaling (MDS) methods were used for grouping of the species. Principal components analysis (PCA) was used to identify the most variable morphological characters. (Podani 2000; Safaei *et al.* 2016). Data analyses were performed by PAST ver. 2.17 (Hammer *et al.* 2012).

Molecular analysis

The obtained ISSR bands were treated as binary characters (presence = 1, absence = 0). The number of

Table 2. Morphological characters and their code.

Character	Code				
Head diameter	x<3	3≤x≤6	x>6		
Flower number	x<80	80≤x≤150	x>150		
Bracts number	x<80	80≤x≤120	x>120		
Appendages length of median bracts	x<9	9≤x≤15	x>15		
Appendages width of median bracts	x< 5	5≤x≤15	x>15		
Crolla length	x< 20	20≤x≤25	x>25		
Habitate	Woodland	Alpine	Stepp		
Leaves indumentum	Present	Absent			
Stem leaves	Interruptedly decurrent	Countinuously decurrent	Undecurrent		
Uppermost leaves	Distant from the head	Close to the head	Surrounding the head		
Appendages	Present	Absent			
Inner bracts indumentum	Smooth	Scabrous			
Position of median bracts	Imbricated	Spreading	Recurved	Spreading-recurved	Imbricated-spreading
Appendages shape of median bracts	Sagitate	Triangular	Rhombic	Ovate	Lanceolate
Appendages margin of median bracts	Smooth	1-2 spins	Spinose		
Receptacle bristles	Smooth	Scabrous			
Corolla color	Yellow	Pink	Purple	White	
Ratio limb to Anther tube	Longer	Shorter	As long as		
Anther tube color	Yellow	Pink	Purple	White	

private bands versus common bands was determined. The genetic diversity parameters like Nei's gene diversity (H), Shannon information index (I), number of effective alleles, and percentage of polymorphism (Freeland *et al.* 2011) were determined for each population. Nei's genetic distance was used for clustering (Weising *et al.* 2005). Neighbor Joining (NJ) and UPGMA (Unweighted paired group using average) clustering were used for the species grouping after 100 times bootstrapping/permutations (Freeland *et al.* 2011). The consensus tree was constructed from the obtained morphological and ISSR trees. Similarly, tree distance was estimated accordingly. The Mantel test between dendrograms was performed to check their agreement. PAST ver. 2.17 (Hammer *et al.* 2012) and DARwin ver. 5 (Perrier & Jacquemoud-Collet 2006) programs were used for these analyses. AMOVA (analysis of molecular variance) (with 1000 permutations) as implemented in GenAlex 6.4 (Peakall and Smouse 2006) was used to determine species genetic differentiation. Gene flow was determined by: (1) calculating Nm an estimate of gene flow from Gst by PopGene ver. 1.32 (1997) as: Nm 1/4 0.5(1 e Gst)/Gst, (2) reticulation analysis that is based on the least square method as performed in T-REX (Boc *et al.* 2012).

RESULTS

Morphometry

UPGMA dendrogram of the studied *Cousinia* species based on morphological characters (Figure 1) placed the studied samples of most of the species together and in a separate sub-cluster. This indicates that *Cousinia* species can be differentiated by the used morphological features. UPGMA dendrogram also separated *Cousinia* species of the two sections *Cynaroideae* and *Platyacanthae*.Therefore, the morphological characters studied can delimit these sections too.

PCA analysis of morphological characters revealed that the first two PCA components comprised about 79% of total variation. Morphological characters like shape and length of the appendages of the median bracts, diameter of the heads, the No. of flowers and length of the corolla had the highest value of correlation with these components and are the most variable morphological features among the studied plants. In fact, these morphological features are of taxonomic value in the two sections *Cynaroideae* and *Platyacanthae*.

ISSR assay

The used ISSR primers produced 36 reproducible bands/loci, out of which only 1 band was monomorphic, while the others were polymorphic bands. The highest number of ISSR bands occurred in *C. keredjensis* Bornm. & Gauba (20), while *C. cynaroides* C. A. Mey had the lowest number of bands (5). A single private ISSR band occurred in *C. keredjensis*, while the other bands were common among the *Cousinia* species.

Discriminating power of ISSR loci as determined by Gst against Nm (migration) analysis (Table 3), revealed that almost all ISSR loci have excellent discriminating power (>0.95). Therefore, ISSR markers are efficient in differentiating *Cousinia* species studied.

The highest value for Nei genetic distance (0.87) occurred between *C. bienerti* Bunge and *C. elwendensis* Bornm., followed by *C. freynii* Bornm. and *C. elwendensis* (0.81). Similarly, the lowest value for the same (0.02) was observed between *C. reshingerorum* Bornm. and *C. bienerti*.

UPGMA dendrogram of the studied *Cousinia* species based on ISSR data (Figure 2) separated these species in distinct sub-clusters. Therefore, ISSR molecular markers can be used in taxonomy of the genus. These molecular markers can also differentiate two sections of *Cynaroideae* and *Platyacanthae*.

AMOVA produced significant genetic difference among the studied *Cousinia* species (P = 0.001), which indicates that the studied species are genetically differentiated. AMOVA revealed that 99% of total genetic difference was due to among species genetic differentiation, while 1% was due to within species genetic variability.

The species relationship illustrated by UPGMA dendrograms based on morphological features and molecular data were not congruent. It was also illustrated in the consensus tree of these dendrograms (Figure 3). This tree revealed that only in some cases the studied *Cousinia* species show the same relationship in both morphological and molecular trees. For instance, *C. zardkuhensis* Attar & Ghahreman (No. 27 in Figure 3) and *C. chlorosphaera* Bornm. (No. 48 in Figure 3) were placed close to each other. The same applied for for *C. platyacantha* Bunge (No. 63 in Figure 3) and *C. freynii* (No. 64 in Figure 3). Similarly, three species of *C. reshingerorum*, *C. bienerti* and *C. trachyphyllaria* Bornm. & Rech. f. (No. 65-67 in Figure 3) formed a distinct cluster in the obtained consensus tree. The rest of *Cousinia* species studied were placed together in an unresolved cluster. This means that, their relationship is differently pictured in the obtained morphological and molecular dendrograms. Tree distance between the obtained morpho-

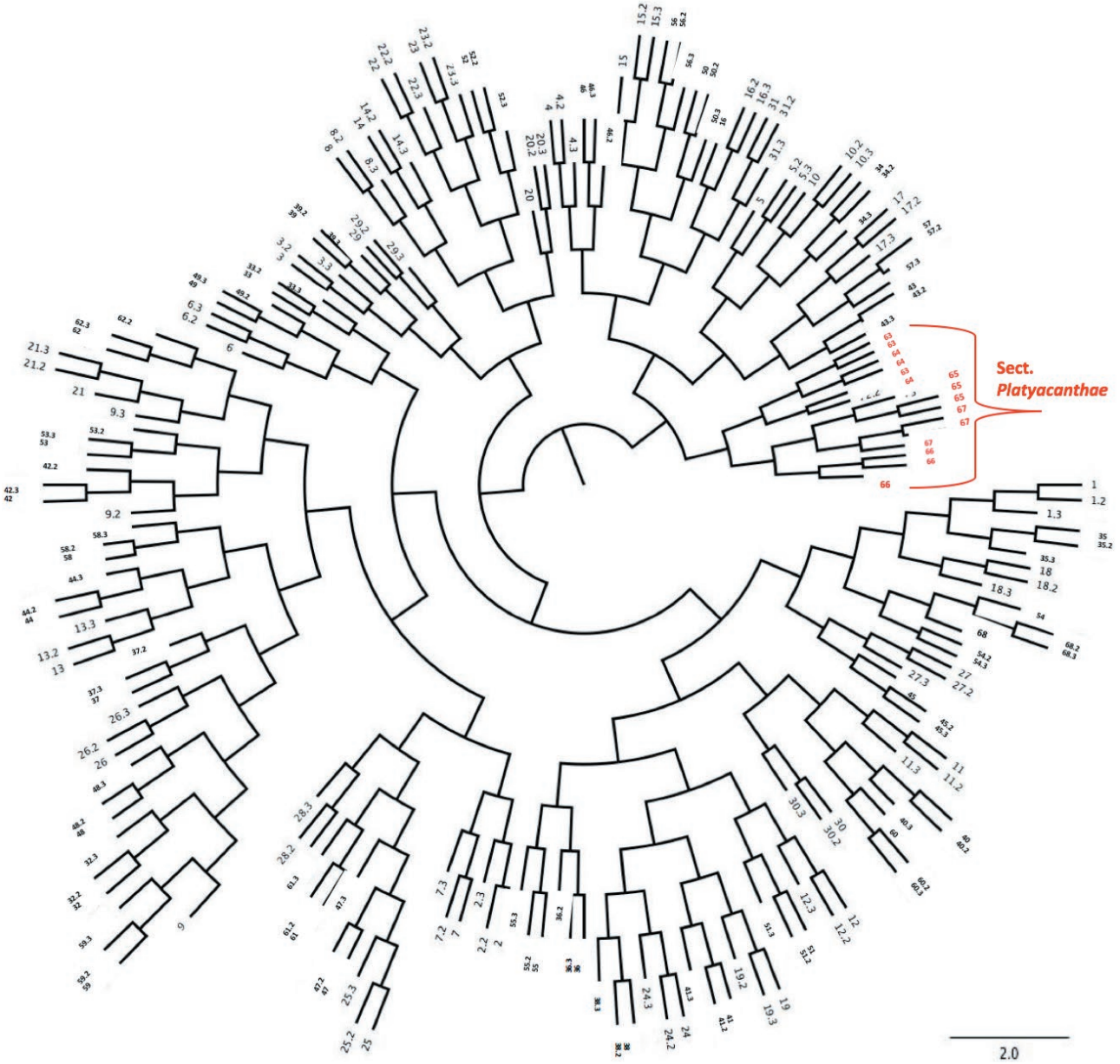

Figure 1. UPGMA dendrogram of the studied *Cousinia* species based on morphological data. (The specie 1-68 are according to Table 1).

logical and ISSR dendrograms after adjusting the edges in each dendrogram was 0.36. Similarly, comparison of these two dendrograms based on Quartet tree distance method, produced 0.64 difference. Both these results indicate that morphological relationship of the studied *Cousinia* species, differed in great extent with ISSR based species relationship.

The performed Mantel test between morphological distance and Nei genetic distance produced non-significant correlation (Correlation R = 0.06, p = 0.113). This indicates that morphological divergence in the studied species is not correlated with genetic distance.

DISCUSSION

As mentioned by the authors, taxonomy and molecular phylogeny of the genus *Cousinia* is complicated and unresolved mainly due to disagreement between the morphological and molecular phylogenetic studies (See

Table 3. Discrimination power of ISSR loci in studied Cousinia species.

Locus	Sample Size	Ht	Hs	Gst	Nm*
Locus1	204	0.1107	0.0000	1.0000	0.0000
Locus2	204	0.4027	0.0000	1.0000	0.0000
Locus3	204	0.4931	0.0000	1.0000	0.0000
Locus4	204	0.2712	0.0000	1.0000	0.0000
Locus5	204	0.3893	0.0000	1.0000	0.0000
Locus6	204	0.0843	0.0000	1.0000	0.0000
Locus7	204	0.0571	0.0000	1.0000	0.0000
Locus8	204	0.3599	0.0000	1.0000	0.0000
Locus9	204	0.3270	0.0000	1.0000	0.0000
Locus10	204	0.4961	0.0000	1.0000	0.0000
Locus11	204	0.3655	0.0088	0.9759	0.0124
Locus12	204	0.2297	0.0000	1.0000	0.0000
Locus13	204	0.4650	0.0000	1.0000	0.0000
Locus14	204	0.2297	0.0000	1.0000	0.0000
Locus15	204	0.3270	0.0000	1.0000	0.0000
Locus16	204	0.1454	0.0088	0.9394	0.0323
Locus17	204	0.2712	0.0000	1.0000	0.0000
Locus18	204	0.1499	0.0132	0.9118	0.0484
Locus19	204	0.0394	0.0088	0.7763	0.1441
Locus20	204	0.0107	0.0088	0.1791	2.2922
Locus21	204	0.0571	0.0000	1.0000	0.0000
Locus22	204	0.1107	0.0000	1.0000	0.0000
Locus23	204	0.2712	0.0000	1.0000	0.0000
Locus24	204	0.4377	0.0000	1.0000	0.0000
Locus25	204	0.4983	0.0000	1.0000	0.0000
Locus26	204	0.1609	0.0000	1.0000	0.0000
Locus27	204	0.2297	0.0000	1.0000	0.0000
Locus28	204	0.2712	0.0000	1.0000	0.0000
Locus29	204	0.0843	0.0000	1.0000	0.0000
Locus30	204	0.2297	0.0000	1.0000	0.0000
Locus31	204	0.2076	0.0000	1.0000	0.0000
Locus32	204	0.0843	0.0000	1.0000	0.0000
Locus33	204	0.0290	0.0000	1.0000	0.0000
Locus34	204	0.0571	0.0000	1.0000	0.0000
Locus35	204	0.1609	0.0000	1.0000	0.0000
Locus36	204	0.0571	0.0000	1.0000	0.0000
Mean	204	0.2270	0.0013	0.9941	0.0030

Nm = estimate of gene flow from Gst or Gcs. E.g., Nm = 0.5(1 - Gst)/Gst.

for example, Sausana *et al.* 2003; Lopez-Vinyallonga *et al.* 2009). Moreover, several overlapping morphological characteristics at the species level makes the species identification and delineation difficult (Attar and Djavadi 2010). In the present study, we could delimit the studied *Cousinia* species based on both the used morphological and molecular data. We suggest that certain morphological characters like shape and the length of the appendages of the median bracts, diameter of the heads, the No. of flowers and the length of the corolla are taxonomically useful at the species level.

Interesting enough, the both sections *Cynaroideae* and *Platyacanthae* are separated from each other due to the difference in traits such as stem leaves and appendages of median bracts. Therefore, these characters are of more practical utility, particularly in sectional level classification in the genus *Cousinia*.

The obtained species relationship based on morphological features are in agreement with previous studies. For example, within the section *Platyacanthae*; *C. Platyacanthae* and *C. freynii* were placed close each other due to the similarity in all features except color of corolla. Their close affinity to each other was also noticed by Asaadi and Mehregan (Flora of Iran 2017). Similarly, *C. reshingerorum*, *C. bienerti* and *C. trachyphyllaria* showed morphological resemblance due to the traits such as No. of the flowers, the length of the corolla, the color of the anther tube, inner bracts, receptacle bristles, diameter of head, ratio of limb/tube. Close morphological affinity among these species was also illustrated by Asaadi and Mehregan (Flora of Iran 2017).

In the section *Cynaroideae*, ISSR data showed genetic affinity between *C. grantii* Rech. f. and *C.grandis* C. A. Mey., and also between *C. verbascifolia* Bunge and *C. monocephala* Bunge. These species also showed morphological similarities. The same holded true for *C. pergamaceae* Boiss. & Hausskn., *C. millefontana* Rech. f., and *C. carolihenrici* Attar & Ghahreman; as well as for *C. zardkuhensis* and *C. chlorosphaera*. ISSR data revealed close affinity between *C. disfulensis* Bornm., *C. jaccobsii* Rech. f. and *C.kermanshahensis* Attar; which is almost in agreement with the morphological data. The same applied for *C. nana* Attar and *C. kotschyi* Boiss. These results are almost in agreement with the taxonomic treatment of the section *Cynaroideae* (Attar and Djavadi 2010). The other studied *Cousinia* species in the section *Cynaroideae* differed in their affinity in genetic tree versus morphological tree. This is in agreement with results of Lopez-Vinyallonga *et al.* (2009), as they also indicated that morphological traits are highly incongruent with molecular data in *Arctium-Cousinia* complex and considered morphological characters homoplasious.

In general, various reasons were suggested for this incongruence between molecular and morphological analyses: the high number of taxa in the genus *Cousinia,* homoplasious of the morphological traits, convergent evolution (Susanna *et al.* 2003; Lopez-Vinyallonga *et al.* 2009), incomplete lineage sorting (Zhang *et al.* 2015); as well as the occurrence of intermediate forms and

Figure 2. UPGMA dendrogram of the studied *Cousinia* species based on ISSR data.

homoploid hybrid speciation, which there is little proof to prove them (Mehregan & Kadereit 2009; Lopez-Vinyallonga *et al.* 2009). Furthermore, the genus *Cousinia* with its relatively young geological age (ca. 8.7 mya) and high number of taxa is thoroughly unusually exposed to speciation (Lopez-Vinyallong *et al.* 2009). This is entirely consistent with the results reported by Lopez-Vinyallonga *et al.* (2009), as they also revealed that the dominant factor in speciation of the genus *Cousinia* is allopatric geographic speciation. These may partly justify the complexity and incongruence of the relationships in the studied species of the genus *Cousinia*.

In conclusion, along with confirmation by the published literature, the current study proved that the morphological characters and ISSR molecular data are useful for the correct identification and species delimitation within the *Cynaroideae* and *Platyacanthae* sections. Both quantitative and qualitative morphological characteristics are important and suitable for the identification of species of the genus *Cousinia*.

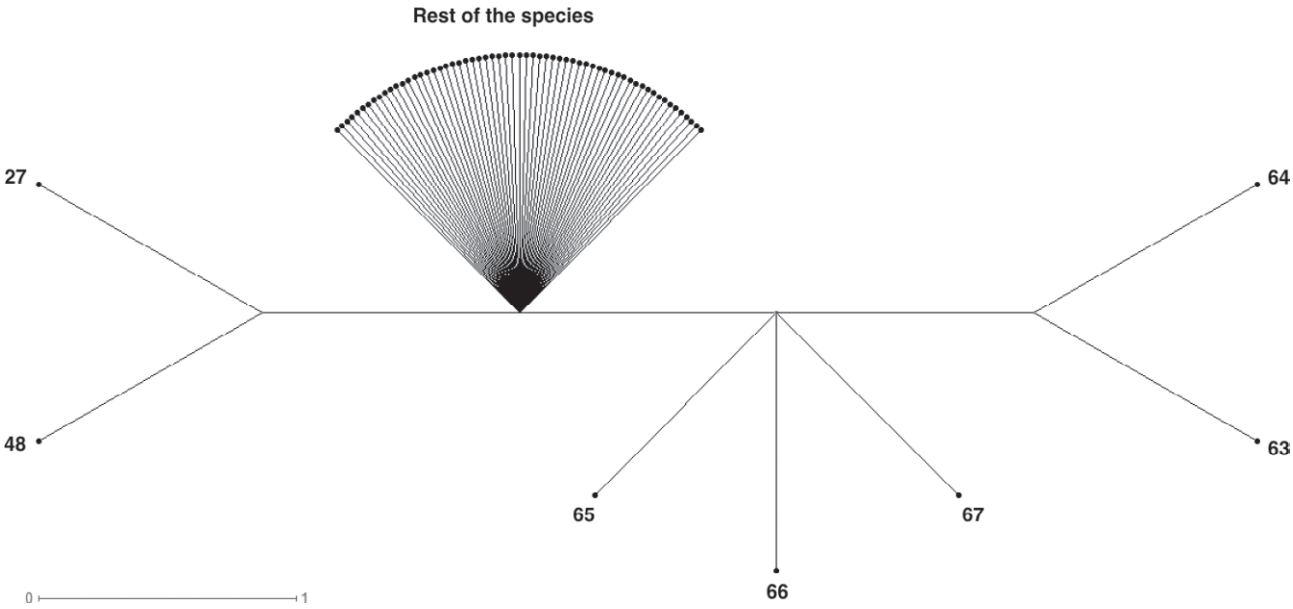

Fig. 3. Consensus tree based on morphological and ISSR dendrograms in studied *Cousinia* species.

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Karyological analysis of twelve *Euphorbia* species from Turkey

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Abstract. Karyotypes of 12 *Euphorbia* species were studied and described for the first time; *Euphorbia cheiradenia, E. pannonica, E. pestalozzae, E. petrophila, E. pisidica, E. thessala* and *E. yildirimli*. Karyological analyses indicate relationships among the species with respect to their asymmetry indices. Most of the investigated taxa are diploids with 2n = 2x = 18. *E. macroclada* and *E. smirnovii* showed tetraploid cytotypes 2n = 4x = 36. All karyotypes are symmetrical, consisting of metacentric and submetacentric chromosomes. The chromosomes range in size from 0.79 µm to 2.20 µm. The total haploid chromosome length (THL) ranges from 8.75 µm (*E. terracina*) to 16.78 µm (*E. petrophila*). Principal Coordinate Analysis with five uncorrelated parameters was performed to determine the karyological relationships among the taxa.

Keywords: chromosome number, karyotype asymmetry, *Pithyusa*, *Esula*, tetraploid, karyosystematics.

INTRODUCTION

Genus *Euphorbia* (Euphorbiaceae), with over 2000 species, is the second-largest genus of flowering plants in the world (Bruyns *et al.* 2006; Horn *et al.* 2012). The use of *Euphorbia* species as medicine and poison are of greatest biocultural importance and the most-valued medicinal use of *Euphorbia* species is in the treatment of digestive and respiratory complaints, inflammation and injuries (Ernst *et al.* 2015). Additionally, *Euphorbia* taxa have been used in Turkish folk medicine to treat rheumatism, swelling and especially as a wart remover (Baytop 1984). There are also various biological effects as antioxidant, antimicrobial and wound healing activities (Barla *et al.* 2006, 2007; Özbilgin *et al.* 2018, 2019).

The genus is represented in Turkey by two subgenera, E. subg. *Chamaesyce* Raf. and E. subg. *Esula* Pers., with a total of 120 taxa (Öztekin 2012; Genç and Kültür 2016). *Euphorbia* subg. *Esula* comprises about 480 species, most of which are annual or perennial herbs (Riina *et al.* 2013). According to the recent classification of Riina *et al.* (2013), the subgenus is represented by 14 sections in the Turkey (Genç and Kültür 2018).

The chromosome numbers in these two subgenera are very variable. Various chromosome numbers have been reported for the subg. *Esula* (2n = 10,

12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 36, 40, 42, 44, 56, 60, 64, 72) and sect. *Pithyusa* (2n = 16, 18, 28, 36, 40, 72) worldwide (Riina *et al.* 2013).

The chromosome numbers of 64 taxa, which are distributed in Turkey naturally have not been determined yet. Up to now, the chromosome numbers of 12 *Euphorbia* taxa were reported from Turkey (Radcliffe-Smith 1976; Strid 1987; Kesercioglu *et al.* 1990; Iyer 1991; Vicens *et al.* 1991; Rice *et al.* 2015; Genç and Kültür 2017). Considering that the number of chromosomes of the vast majority of these species is not yet clear, there is a remarkable gap in this area.

This study aimed to determine the chromosome numbers and karyomorphology of 11 perennial *Euphorbia* sect. *Pithyusa* species distributed in Turkey. In addition, *E. terracina* (sect. *Pachycladae*) was also included in the present study as an external group.

MATERIALS AND METHODS

Twelve *Euphorbia* species were analysed in this study. Seeds of 12 species were collected from mature capsules in their natural habitats as given in Table 1. Voucher specimens are deposited in İstanbul University, Faculty of Pharmacy Herbarium (ISTE). For each population, 9-27 mature seeds were selected from at least 5 individuals (inflorescence). The seeds germinated on wet filter paper in petri dishes at room temperature.

The root tips meristems were excised at 9-9.30 am. Mitotic chromosomes were prepared from root tips and pre-treated with 8-Hydroxyquinoline at +4 °C for 24 h. Roots were fixed for a minimum of 2 h in absolute ethanol:glacial acetic acid, (3:1,v/v), hydrolysed at 60 °C in 1 N HCl for 15 min and stained with Feulgen reagent. Finally, root tips were squashed in a drop of 1% aceto-orcein. Permanent microscopic slides were prepared with Entellan (Rapid Mounting Medium / Merck Darmstadt Germany). Microphotographs of good quality metaphase plates were taken using an Olympus BX53 (Tokyo, Japan) microscope equipped with a high-resolution digital camera. Metaphase observations and chromosome measures were made using the image analysis systems Kameram (Argenit Microsystems, İstanbul, Turkey). The chromosome number and karyotype details were studied in five to fourteen well-spread metaphase plates from different individuals; mean values were used for the analysis.

Chromosome pairs were identified and arranged on the basis of chromosome/chromatid length and any other evident karyomorphological features. The nomenclature used for describing karyotype followed Levan *et*

Table 1. Localities and voucher data of *Euphorbia* taxa examined in the present study.

Species	Voucher specimens	Previous counts (Rice *et al.* 2014)
E. cheiradenia Boiss. & Hohen.	Şanlıurfa, Tektek Mountain National Park, 460m, 18.6.2015, İ.Genç *2398,* Ş.Kültür.	--
E. macroclada Boiss.	Eskişehir-Antalya road, roadsides, 10.8.2015, İ.Genç *2472.*	18
E. niciciana Borbás ex Novák	Karabük, Bolkuş village, 220 m, 03.vi.2015, İ.Genç *2290, G. Ecevit Genç.*	18, 18+1
E. pannonica Host	İstanbul, Dağyenice-Kalfaköy, roadsides, 110m, 18.vii.2016, İ.Genç *2477, G. Ecevit Genç.*	--
**E. pestalozzae* Boiss.	Antalya, Saklıkent, 1780m, 30.vii.2016, İ.Genç *2499.*	--
E. petrophila C.A.Meyer	Kastamonu, Kastamonu-Sinop roadsides, 720m, 14.vii.2015, İ.Genç *2419, G. Ecevit Genç.*	--
**E. pisidica* Hub.-Mor. & M.S.Khan	Burdur, Altınyayla-Gölhisar road, Dirmil pass, 1585m, 23.vi.2014, İ.Genç *2114, G. Ecevit Genç.*	--
E. seguieriana Necker	Ağrı, between Doğubeyazıt-Çaldıran, roadsides, 2600m, 24.vii.2015, İ.Genç *2453.*	16, 18, 40
E. smirnovii Geltman	Erzincan, Ergan mountain, 1510m, 25.vii.2015, İ.Genç *2468, A. Kandemir.*	18 (Genç & Kültür 2017)
E. thessala (Form.) Degen & Dörf.	Kırklareli, Kırklareli-Pınarhisar roadside, 190m, 5.vi.2015, İ.Genç *2304, G. Ecevit Genç.*	--
**E. yildirimlii* Dinç	Eskişehir, Sivrihisar, Aşağıkepen village, gypsum slopes, 900m, 26.viii.2014, İ.Genç *2267, G. Ecevit Genç.*	--
E. terracina L.	Bursa, Mudanya, Söğütlüpınar village, s.l., 7 vıı 2014, İ.Genç *2192, S. Yüzbaşıoğlu*	18, 20, 36

* The species endemic to Turkey.

Table 2. PCoA graphic symbols of some important taxonomic characteristics for *Euphorbia*.

	Seed surface smooth	Seed surface not smooth
Cyathial glands horned	●	■
Cyathial glands hornless	●	■

al. (1964). To determine the karyological relationships among taxa, we performed Principal Coordinate Analysis (PCoA) with five uncorrelated parameters as suggested by Peruzzi and Altınordu (2014). These parameters are basic chromosome number (x), total haploid length (THL), mean centromeric asymmetry (M_{CA}) it is calculated as the mean (L-S)/(L+S)×100 where, for each chromosome, L is the length of long arm and S is the length of short arm; coefficient of variation of chromosome length (CV_{CL}=(L+S)×100) and coefficient of variation of centromeric index (CV_{CI} =S/(L+S) ×100) (Paszko 2006; Peruzzi *et al.* 2009; Zuo and Yuan 2011; Peruzzi and Eroğlu 2013). The software Past 3.03 (Hammer *et al.* 2001; Hammer 2018) was used to perform this analysis.

Seed and gland morphology in the classification of the genus *Euphorbia* is one of the most important taxonomic characters. Therefore, in order to evaluate the resulting PCoA graphic in terms of the seed and gland morphological characters, some symbols are given to the studied species (Table 2).

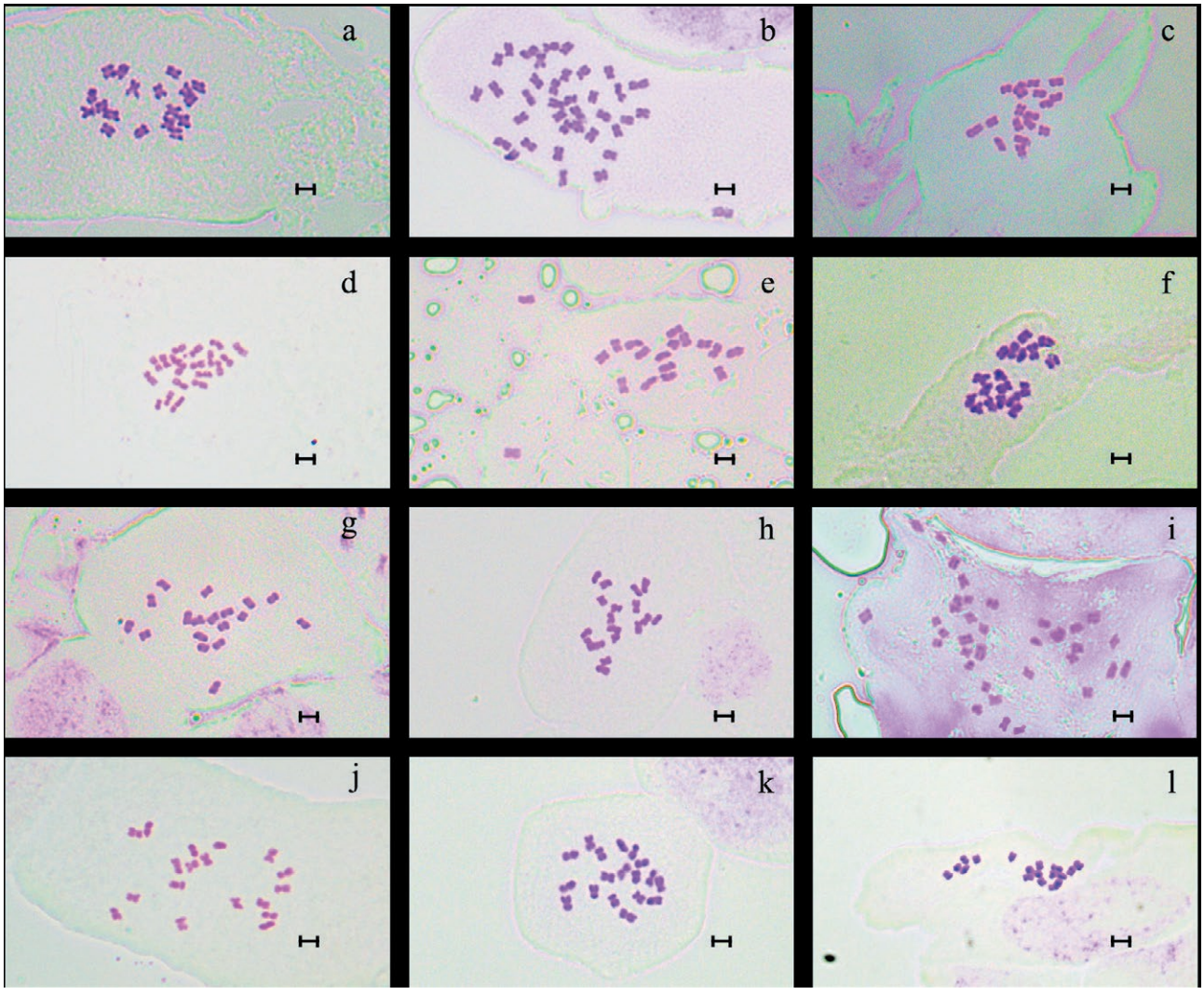

Figure 1. Somatic chromosomes of the studied taxa. (a) *E. cheiradenia*; (b) *E. macroclada*; (c) *E. niciciana*; (d) *E. pannonica*; (e) *E. pestalozzae*; (f) *E. petrophila*; (g) *E. pisidica*; (h) *E. seguieriana*; (i) *E. smirnovii*; (j) *E. thessala*; (k) *E. yildirimli*; **(l)** *E. terracina.* (Scale bars 2 *μm*).

Mitotic metaphase chromosomes are given in Figure 1. Ideograms of these taxa are arranged in order of centromere position and then decreasing the length of homologue chromosome pairs (Figure 2).

RESULTS

Karyomorphological details (shortest chromosome length; longest chromosome length; mean long arm length; mean short arm length; karyotype formula) of 12 species of *Euphorbia* are listed in Table 3. The chromosome numbers, total haploid length, mean centromeric asymmetry, coefficient of variation of chromosome length and the coefficient of variation of centromeric index of the species are also summarized in Table 4. Metaphase plates and their related ideograms of the studied species are presented in Figures 1 and 2.

Karyotype analysis revealed the basic chromosome number x=9 for all species. Most of the species showed diploid cytotypes 2n = 18 but *E. macroclada* and *E. smirnovii* showed tetraploid cytotypes 2n = 4x = 36 (Figure 1).

The chromosomes were mainly metacentric with centromeres localized in the median position. The karyotypes of four species (*E. cheiradenia, E. macroclada, E. smirnovii, E. yildirimli*) included one submetacentric chromosome. Only karyotype of *E. petrophila* included two submetacentric chromosomes.

The ratio of the shortest and the longest chromosome lengths ranged from 0.79 µm (*E. smirnovii*) to 2.20 µm (*E. petrophila*). The ratio of the shortest and the longest total haploid chromosome length (THL) ranged from 8.75 µm to 16.78 µm in *E. terracina* and *E. petrophila,* respectively. The smallest mean short arm length (q) was observed in *E. terracina* (0.44 µm) and *E. seguieriana* (0.51 µm), the largest mean long arm length (p) was observed in *E. petrophila* (1.07 µm) (Table 3).

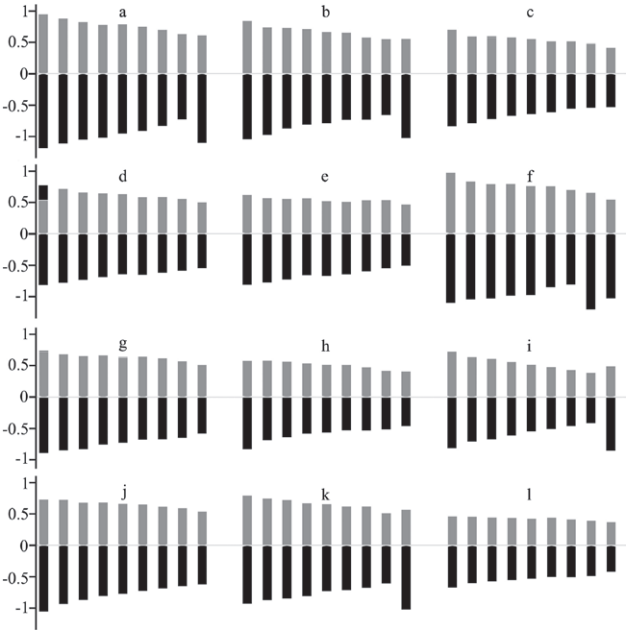

Figure 2. Haploid ideograms of the studied taxa. (a) *E. cheiradenia*; (b) *E. macroclada*; (c) *E. niciciana*; (d) *E. pannonica*; (e) *E. pestalozzae*; (f) *E. petrophila*; (g) *E. pisidica*; (h) *E. seguieriana*; (i) *E. smirnovii*; (j) *E. thessala*; (k) *E. yildirimli*; (l) *E. terracina.*

Table 3. Karyotype analysis of the investigated *Euphorbia* taxa.

	SC–LC	q (µm) Mean (±SD)	p (µm) Mean (±SD)	p+q Mean (±SD)	Karyotype formula
E. cheiradenia	1.35–2.12	0.76(±0.10)	0.98(±0.14)	1.75(±0.22)	16 m + 2 sm
E. macroclada	1.21–1.89	0.67(±0.09)	0.85(±0.13)	1.53(±0.20)	32 m + 4 sm
E. niciciana	0.94–1.53	0.55(±0.08)	0.65(±0.10)	1.20(±0.17)	18 m
E. pannonica	1.05–1.60	0.63(±0.08)	0.68(±0.08)	1.31(±0.16)	18 m
E. pestalozzae	0.98–1.44	0.54(±0.04)	0.67(±0.09)	1.21(±0.13)	18 m
E. petrophila	1.59–2.20	0.79(±0.12)	1.07(±0.12)	1.86(±0.18)	14 m + 4 sm
E. pisidica	1.10–1.64	0.63(±0.06)	0.75(±0.10)	1.38(±0.16)	18 m
E. seguieriana	0.88–1.42	0.51(±0.06)	0.61(±0.11)	1.11(±0.16)	18 m
E. smirnovii	0.79–1.52	0.52(±0.10)	0.62(±0.14)	1.14(±0.22)	32m + 4 sm
E. thessala	1.15–1.78	0.65(±0.06)	0.79(±0.13)	1.44(±0.19)	18 m
E. yildirimli	1.13–1.74	0.66(±0.08)	0.80(±0.13)	1.47(±0.18)	16 m + 2 sm
E. terracina	0.80–1.14	0.44(±0.03)	0.54(±0.07)	0.97(±0.09)	18 m

Abbreviations:**SC**: the shortest chromosome length; **LC**: the longest chromosome length; **p**: mean long arm length; **q**: mean short arm length; **SD**: standard deviation; m: metacentric; sm: submetacentric.

Table 4. Chromosome numbers and karyo-morphometric parameters, symmetry indices for investigated taxa.

	2n	x	THL	M_{CA}	CV_{CL}	CV_{CI}
E. cheiradenia	18	9	15.71	12.56	12.51	6.82
E. macroclada	36	9	13.73	11.37	12.89	7.73
E. niciciana	18	9	10.78	8.43	14.61	3.34
E. pannonica	18	9	11.77	4.17	12.40	1.53
E. pestalozzae	18	9	10.86	10.52	10.87	5.16
E. petrophila	18	9	16.78	14.90	9.49	10.44
E. pisidica	18	9	12.41	8.06	11.25	2.99
E. seguieriana	18	9	10.01	8.49	14.33	4.73
E. smirnovii	36	9	10.30	7.71	19.65	7.96
E. thessala	18	9	12.93	9.56	13.16	4.64
E. yildirimli	18	9	13.21	9.37	12.35	7.54
E. terracina	18	9	8.75	9.85	9.76	3.93

Abbreviations: **THL**: total haploid length; **M_{CA}**: mean centromeric asymmetry; **CV_{CL}**: coefficient of variation of chromosome length; **CV_{CI}**: coefficient of variation of centromeric index.

Euphorbia petrophila, with relatively high intrachromosomal (M_{CA}= 14.90) and *E. pannonica* (4.17) with low intrachromosomal asymmetry, also *E. smirnovii* with high interchromosomal (CV_{CL}=19.65) and *E. petrophila* (9.49) low interchromosomal asymmetry were observed. Intrachromosomally, seed surface smooth and cyathial glands horned species are more asymmetrical than seed surface smooth and cyathial glands hornless species (Table 4).

Twelve *Euphorbia* species were analysed by PCoA (cumulative variance explained by the first two axes: 88.21%). And, the species with the same seed and cyathial gland morphology tend to cluster together except *E. smirnovii* (Figure 3). The most important characters in recognizing these groups as distinct resulted THL, M_{CA} and CV_{CL}.

DISCUSSION

The number, size, and asymmetry of chromosomes are important characteristics that help explain the phylogenetic relationships of species (Eroğlu *et al.*, 2013).

Karyotype data for seven taxa (3 endemic) are reported for the first time in the present study.

Various chromosome numbers have been reported for the sect. *Pithyusa* (2n=16, 18, 28, 36, 40, 72) (Riina et al 2013). However, most of the investigated taxa in this study have the same chromosome number (2n=18), only *E. macroclada* and *E. smirnovii* showed tetraploid cytotypes 2n = 4*x* = 36. According to Riina *et al.* (2013) chromosome number of *E. terracina* is 18 and our results supported this report. However, even though *E. terracina* has 18 chromosomes, it differs from the other investigated taxa in smaller chromosomes (THL=8.75).

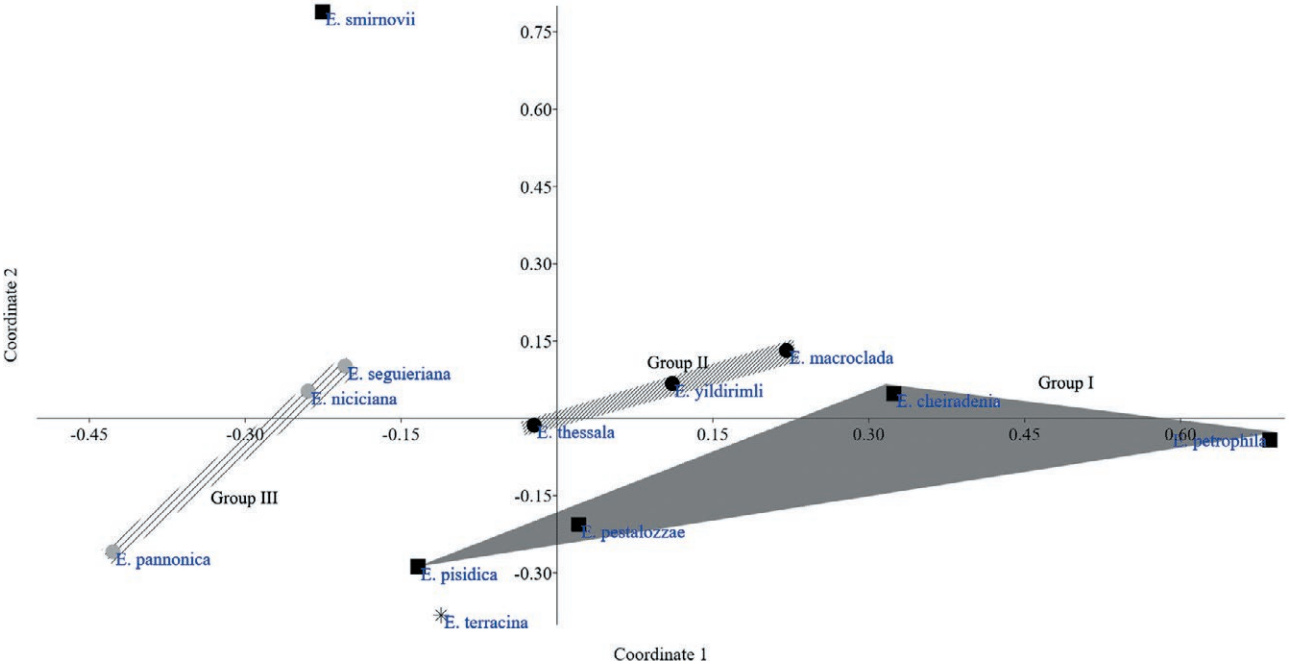

Figure 3. PCoA analysis based on five quantitative karyological parameters of investigated taxa.

The chromosome number of *E. macroclada* was reported 2n = *2x* = 18 by Lessani and Chariat-panahi (1979) and Chariat-Panahi *et al.* (1982). Tetraploid cytotype is reported for the first time for this species. Different chromosome numbers were reported for *E. seguieriana* subsp. *seguieriana* (2n= 16, 18, 40) (Rice *et al.* 2015). In this study, the chromosome number of this species is reported to be 2n = 2*x* = 18.

Euphorbia terracina is a bit separated from other taxa according to the PCoA analysis. So, our results support the position of *E. terracina* in a distinct section (E. sect. *Pachycladae*), as suggested by Riina *et al.* (2013).

Three groups are examined in PCoA graph as **group I** (taxa with seed surface not smooth and cyathial glands horned), **group II** (taxa with seed surface smooth and cyathial glands horned) and **group III** (taxa with seed surface smooth and cyathial glands hornless). But *E. smirnovii* is also partially out of these groups (Figure 3).

As a conclusion, further studies on the chromosome morphology of *Euphorbia* taxa should be performed. Thus, it can be seen more clearly how species are grouped according to the chromosome morphology. In our opinion, studies on the chromosome morphology of *Euphorbia* will contribute to the taxonomy of the genus.

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Investigation of benzyl benzoate toxicity with anatomical, physiological, cytogenetic and biochemical parameters in *in vivo*

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Abstract. In this study, the toxic effects of benzyl benzoate, which is widely used in the food, cosmetics, agriculture and pharmaceutical sectors, have been investigated using *Allium cepa* L. test material. In the determination of toxicity, physiological parameters with determination of root lengths, weight gains and germination percentages; cytogenetic changes with determination of chromosomal abnormalities formation, micronucleus (MN) frequency and mitotic index ratio (MI); anatomical changes with determination of anatomical differentiations in root tip cells; biochemical changes with lipid peroxidation and antioxidant enzyme analysis were determined and the obtained data were evaluated statistically. The bulbs were divided into four groups consisting of one control and three application groups, bulbs of the control group were treated with tap water and the bulbs of the application groups were treated with Benzyl benzoate at doses of 10,000, 25,000 and 50,000 mg/L for 72 hours. At the end of the study, it was determined that germination percentage, weight gain and root length and MI ratio decreased, chromosomal abnormalities, MN formation, MDA, SOD and CAT levels increased dose-dependent in the application groups when compared with the control group. Depending on the application, it has been determined that root cells have chromosomal abnormalities such as fragments, sticky chromosomes, chromosome bridges, unequal distribution of chromatins and c-mitosis. Furthermore, when compared with the control group, it was determined that benzyl benzoate administration caused anatomical changes in root tip cells. It was determined that these changes were in the form of necrosis, cell deformation, flattened cell nuclei, cortex cell deformation, accumulation of certain substances in cortex cells, wall thickening in cortex cells and unclear vascular tissue. In conclusion, it was determined by physiological, anatomical, cytogenetic and biochemical parameters that benzyl benzoate showed a dose-dependent toxic effect in *Allium cepa* L. root cells. Also, the parameters used in the study were determined to be useful biomarkers for the determination of toxicity.

Keywords: benzyl benzoate, *Allium cepa* L., toxicity, chromosomal abnormalities, lipid peroxidation, antioxidant enzymes.

INTRODUCTION

With the increase in the world population, it is rapidly increasing in consumption in many areas such as food, medicine, clothing and cosmetics. Due to this increase, production quantities of foodstuffs, medicines and cosmetic products, that have an important place in human life, are increasing day by day. These increases in production and consumption cause competition among the manufacturers, that encourages the use of various chemicals to companies who want to gain more profit and find faster solutions to some problems occurring in production, marketing and storage. The negative effects caused by the chemicals used are not investigated sufficiently and the damages that can be given to the ecosystem, especially the human health and environment, are ignored (Searle, 1995).

Organisms are exposed to many foreign substances and are commonly referred to as xenobiotics. Exposure of people to xenobiotics can be caused by food additives, cosmetics and medicines that are directly consumed by people and used in the substances, while indirect exposure may be due to pesticides and agricultural drugs used in agricultural control (Akay, 2004; Alam and Jones, 2014).

The increasing use of chemical substances increases the importance of toxicological studies. It is possible to examine the possible effects of chemical substances that can be absorbed by respiration, nutrition or skin through toxicological researches, to develop strategies for control purposes and to prohibit use (Vural, 2005).

Benzyl benzoate is a chemical compound which is widely used in food, cosmetics, agriculture and pharmaceutical sectors. It is also widely used in combating mites and insects. The aim of this study was to investigate the physiological, cytogenetic, anatomical and biochemical effects of benzyl benzoate that is one of the most frequently used chemical substances, on *Allium cepa* L.

Benzyl benzoate is an ester of benzyl alcohol and benzoic acid, also known as benzoic acid phenylmethyl ester, benzoic acid benzyl ester, benzyl benzene carboxylate, benzyl phenyl formate and phenylmethyl benzoate. It is a chemical compound with the closed formula $C_{14}H_{12}O_2$, open formula $C_6H_5COOCH_2C_6H_5$, molecular weight 212.25 g/mol, density 1.118 g/cm^3, melting point 18-20 °C and boiling point 323 °C (Hassan and Mossa, 1981; Ash and Ash, 2004). It has a sharp burning taste and is colorless, oily and liquid. Benzyl benzoate is rapidly hydrolyzed to benzyl alcohol and benzoic acid in vivo, and benzyl alcohol is then oxidized to glycine-conjugated benzoic acid to form hippuric acid (Hassan and Mossa, 1981). Benzyl benzoate can be formed as a result of condensation of benzyl alcohol and benzoic acid and also from benzaldehyde by Tishchenko reaction (Kamm and Kamm, 1941). It is naturally found in various essential oils with Peru and Tolu balsams. In addition, it has been determined that benzyl benzoate was found to be 89.5% in the oils obtained from the leaves of the *Cinnamomum sulphuratum* Nees plant and 98.2% in the root crusts oils (Kar, 2003; Rameshkumar and George, 2006).

Benzyl benzoate has a wide variety of application areas. Used as a diluent and solvent in solid aromatics, as a stabilizer in perfume compositions due to its low volatility, as solvent for substances such as cellulose acetate and nitrocellulose, instead of camphor in cellulose and plastic pyroxylin compounds and also in many different sectors and products such as confectionery and chewing gum products (Kar, 2003). Benzyl benzoate can also be used as an additive in foods. Benzyl benzoate use has been approved by the EU Food Processing Agents as a sweetening food additive (EU, 2012), and carrier solvent by the FAO/WHO Expert Committee on Food Additives (JECFA, 1996). It is also known that benzoic acid and its compounds are used in foods such as chocolates, beverages, oils, sauces, milk powders, fats, ketchup, mayonnaise, bakery products, dry yeasts, sugars, gums, salads and cookies (Erkmen and Bozoğlu, 2008). Benzyl benzoate is also known to be used in the cosmetic industry. In a study conducted in England, it was reported that benzyl benzoate was found in 23% of the cosmetics examined in research (Buckley, 2007). Benzoic acid and its species are generally used as pH adjuster and preservative in cosmetic products (Wenninger *et al.*, 2000). Benzyl benzoate is also used to increase agricultural yield by combating pests in agricultural production. Benzyl benzoate has been reported to be acaricidal (McDonald and Tovey, 1993) and insecticidal (Jantan *et al.*, 2005). One of the most common uses of benzyl benzoate is the health field. It is widely used to combat lice and to treat scabies. Benzyl benzoate is topically applied for the treatment of scabies. It should be applied to all skin surfaces from the scalp to the soles of the skin during the treatment process and should be avoided from contact with the eye and its use in inflamed or cracked skin. If used in children may show an irritant effect (Stuart *et al.*, 2009). Benzyl benzoate has been in use in the treatment of scabies since 1937. It is formulated as emulsions in concentrations from 20% to 35%. As a complaint after application, there is burning, irritation and tenderness in the case of intense contact. It may cause irritation dermatitis, especially in the genital area and on the face (Campbell and Rew, 1986; Habif, 2016).

The mechanism of action of benzyl benzoate against the disease agent *Sarcoptes scabiei* L. is not yet known (Micali and Lacarrubba, 2016). When used topically, it is a relatively toxic compound. Although its chronic effects are not known, it may cause mild allergic reactions which may be lost after the end of the exposure. When used as an acaricide, it may cause diarrhea, peristalsis, enterospasm, intestinal colic, spastic constipation, pylorospasm, hypertension, contraction of seminal vesicles and bronchospasm in the intestines (Wexler *et al.*, 2005). A 7-year-old male patient was reported to have died after marrow transplantation for aplastic bone marrow disease. The death cause could not be determined precisely, but the month before the diagnosis, his body was washed with ethyl alcohol, water, polysorbate and Ascabiol (containing 10% benzyl benzoate and 2% disulfiram) and death was probably caused by the chronic overdose of the scabicide (Hayes and Laws, 1991).

It was reported that oral LD 50 values of benzyl benzoate varied from 1700 mg/kg in rats to 22440 mg/kg in dogs. When applied to animals too often or over a large area of the skin, saliva can trigger signs of systemic toxicity such as pylorization, incoordination of muscles, tremors, the progression of hind limbs, severe convulsions, shortness of breath and death. When administered in high doses to laboratory animals, it may cause incoordination, hyperexcitation, convulsions, ataxia and respiratory paralysis (Wexler *et al.*, 2005). In addition, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) has determined the ADI (Average Daily Intake) value of benzyl benzoate as 5 mg/kg, indicating the amount that a person can consume without a health risk throughout his or her life, based on the body weight of the individual (WHO, 2001).

In this study, *Allium cepa* L. test was used to investigate the effects of benzyl benzoate. *Allium cepa* L. test was considered to be suitable for evaluating chromosome damage and changes in the mitotic cycle because of the small number of chromosomes (2n = 16) and the large structure. Analysis of chromosomal changes allows the determination of structural changes, however, it is achievable to observe the numerical changes occurring in chromosomes. In addition, it has many advantages such as low cost, multiple roots, short test duration, storage and ease of use, ease of observing nucleus and abnormal events (Fiskesjö, 1985; Liu *et al.*, 1995). The data obtained as a result of the *Allium cepa* L. test provide accurate estimates of the effects of the agent investigated on other living biodiversity. Cytotoxicity tests using in vivo plant testing systems, such as *Allium cepa* L., have been approved by several researchers performing in vitro and in vivo animal testing and the results obtained are similar (Vicentini *et al.*, 2001; Teixeira *et al.*, 2003). In the evaluations, 76% of 148 chemicals evaluated with *Allium cepa* L. test gave positive results, this test was accepted as a standard test to determine the chromosomal damage caused by chemicals (Grant, 1982). Applying chemicals to *Allium cepa* L. root tips, cell progression can be blocked at one of the stages of the cell cycle or cell division. The applied chemical has a toxic effect by causing chromosomal abnormalities, bridge and MN formation in the root tip cells (Bonciu *et al.*, 2018).

MATERIALS AND METHODS

Preparation of research materials and application groups

This study was carried out using benzyl benzoate at doses of 10,000 mg/L, 25,000 mg/L and 50,000 mg/L. Approximately equally large and healthy *Allium cepa* L. bulbs are used as research material. The bulbs were divided into four groups of one (1) control, three (3) administration groups and placed in glass beakers 85x100 mm in diameter and allowed to germinate at room temperature for 72 hours. During the application period, the bulbs in the control group were treated with tap water and the bulbs in the application groups were treated with benzyl benzoate at doses of 10,000 mg/L, 25,000 mg/L and 50,000 mg/L. At the end of the period, the root ends were washed with distilled water and prepared for cytogenetic analysis using standard crushing preparation techniques (Qian, 2004).

Measurement of physiological parameters

At the end of the application period, the root lengths were determined with the millimetric ruler based on the radicular formation of the bulbs germinating and the weight gains were determined by the precision scales by taking into consideration the weight differences obtained before and after the application. Germination percentages were determined using equation 1.

$$\text{Germination Percentage} = \frac{\text{Number of Germinated Bulbs}}{\text{Total Number of Bulbs}} \times 100 \quad (1)$$

Chromosomal abnormalities, micronucleus (mn) test and mitotic index (mi) determination

The root tips cut about 0.5 cm in length were fixed for two hours at the 'Clarke' fixator, washed for 15 min-

utes in 96% ethanol and stored in +4° C at 70% ethanol. In the next step, the root tips were hydrolyzed in 1N HCl for 17 minutes at 60 ° C, incubated in 45% acetic acid for 30 minutes and stained with Acetocarmine for 24 hours, then crushed with 45% acetic acid and photographed at X500 magnification in a binocular research microscope (Qian, 2004; Staykova *et al*., 2005). The criteria determined by Fenech *et al.* (2003; 2010) were taken into consideration in the determination of the existence of MN.

Prepared preparations were examined in a binocular research microscope to determine Mitotic Index (MI) ratio and the percentage of MI was determined by using equality 2.

$$\text{Mitotic Index (\%)} = \frac{\text{Number of Divided Cells}}{\text{Total Number of Analyzed Cells}} \times 100 \quad (2)$$

Anatomical damages

In order to determine the anatomical damages in the root tip meristematic cells, the *Allium cepa* L. root ends were washed with distilled water and the cross-sections were taken and stained with methylene blue.

Biochemical analysis

Determination of lipid peroxidation

Lipid peroxidation measured according to the method specified by Ünyayar *et al.* (2006) measuring the amount of malondialdehyde (MDA). The values of the MDA content are taken from the measurements of three independent samples and are expressed as mean + standard error (SE) µmol/g fresh weight (FW).

Antioxidant enzyme analysis

Approximately 0.5 g of the tissue sample taken from the control and administration group root tips were cut into small pieces by washing with deionized water and homogenized by trituration with 5 mL chilled sodium phosphate buffer. The homogenates were transferred to new tubes and centrifuged at 10,500 rpm for 20 minutes at room temperature, and the supernatant was stored at +4 ° C for antioxidant enzyme analysis.

Superoxide dismutase (SOD) determination

Superoxide Dismutase (SOD) activity was determined by making some modifications to the method of Beauchamp and Fridovich (1971). One unit SOD enzyme activity was determined as the amount of SOD enzyme required for 50% inhibition of NBT reduction under application conditions. The values of the SOD content were taken from the measurements of three independent samples and are expressed as mean + standard error (SE) U/mg fresh weight (FW).

Catalase (CAT) determination

Catalase activity (CAT) was determined according to the method determined by Beers and Sizer (1952). The CAT activity unit was defined as 0.1 unit change at 240 nm absorbance. The values of the CAT content were taken from the measurements of three independent samples and expressed as mean ± standard error (SE) OD_{240nm}/min.g fresh weight (FW).

Statistical analysis

SPSS Statistics V 23.0 (IBM Corp., USA, 2015) package program was used for the analysis of statistical data. The statistical differences between the groups were evaluated by using One-way ANOVA and Duncan tests. Data were given as mean ± SD values and P value was considered as statistically significant when less than 0.05.

RESULTS AND DISCUSSION

The effects of benzyl benzoate on germination percentage, root length and weight gain are shown in Table 1. Treatment of the *A. cepa* test material with different doses of benzyl benzoate showed a germination inhibitory effect. The germination percentage was 100% in Group I, 83% in Group II, 63% in Group III and 47% in Group IV. It was determined that the germination per-

Table 1. Effects of different doses of benzyl benzoate on germination percentage, root length and weight gain.

Groups	Germination Percentage (%)	Average Root Length ±SD	Weight gain (g)
Group I	100	8.25±0.81[a]	+7.71
Group II	83	5.58±0.75[b]	+4.88
Group III	63	3.13±0.60[c]	+2.96
Group IV	47	1.30±0.37[d]	+0.44

*Group I: Control, Grup II: 10,000 mg/L benzyl benzoate, Grup III: 25,000 mg/L benzyl benzoate, Grup IV: 50,000 mg/L benzyl benzoate. Means with the different letters are statistically significant (P<0.05).

centage decreased with the increase in the benzyl benzoate doses. Although there is no comprehensive study investigating the effects of benzyl benzoate on root length in the scientific literature, there are similar studies conducted with other food additives and chemicals that support the results. For example, in a study conducted by Singh (2017), tartrazine, which is used as a coloring additive in food, medicine and cosmetic fields, was applied to the *Vigna radiata* (L.) R. Wilczek seeds at 0.05 ppm and 0.1 ppm doses, consequently 90% germination occurred in the 0,05 ppm tartrazine treated group, 0.1 ppm tartrazine treated group is not germinated and decrease in germination percentage due to application doses. Bidlan *et al.* (2004) investigated the effects of different doses of hexachlorocyclohexane on germination in radish and mung bean. As a result, the percentage of germination for both species was reported to decrease due to the application dose.

Benzyl benzoate also showed an inhibitory effect on another physiological parameter, root length. With the increase in the dose of benzyl benzoate, it was determined that the root length decreased. The average root lengths of the groups were measured as 8.25±0.81 cm in Group I, 5.58±0.75 cm in Group II, 3.13±0.60 in Group III and 1.30±0.37 in Group VI. The maximum root length was determined in the control group and the lowest root length was determined in Group IV treated with a 50,000 mg/L dose of benzyl benzoate. There are similar studies conducted with other food additives and preservatives by other researchers that support our findings. In a study conducted by Adeyemo and Farinmade (2013), monosodium glutamate (MSG), which is used as a flavor-enhancing additive in foods, is given to *A. cepa* test material with doses of 1.0 g/L, 3.0 g/L, 5.0 g/L and 7.0 g/L, changes in the root length were measured for five days. As a result, it was reported that MSG application inhibits root growth at all test concentrations and this is statistically significant at higher doses. In another study by Koç and Pandir (2018), *A. cepa* test material was treated with different doses of sunset yellow (E-110) and brilliant blue (E-133) additives, which were used as a coloring agent in foodstuffs. It was reported that root lengths decreased due to increasing application dose.

In the study, it was determined that benzyl benzoate application had an inhibitory effect on weight gain which is another physiological parameter. At the end of the 72 hours application period, the mean weight gains were measured as 7.71 g in Group I, 4.88 g in Group II, 2.96 g in Group III and 0.44 g in Group IV. It was determined that there was an inverse ratio between the dose of benzyl benzoate administered and weight gain. Although there is no comprehensive study investigating the effects of benzyl benzoate on weight gain, there are similar studies in the treatment of some diseases and chemical substances used in the fight against insects. In a study conducted by Arslanoglu (2011), *A. cepa* test material was administered Basudin 60 EM insecticide at doses of 600, 1200 and 1800 ppm, resulting in a reduction in weight gain due to increased dose. In another study, Çavuşoğlu *et al.* (2012b) applied 100 mg/kg, 250 mg/kg and 500 mg/kg doses of thiamethoxam insecticide to the *A. cepa* test material and reported that the weight gain was reduced depending on the application doses.

In our study, cytogenetic effects of benzyl benzoate application were investigated by determining chromosomal damages, MN formation and MI ratio. The effects of benzyl benzoate on MN formation are shown in Table 2 and Figure 1. Benzyl benzoate was found to promote the formation of MN in root tip cells of *A. cepa* depending on the dose of administration. As a result of microscopic examinations, there was an average of 0.30 ± 0.48 MN formation in the control group. In Group I, which is the control group, only a few MN formation was observed and it was determined that MN formation increased with increasing dose of benzyl benzoate. The mean MN formation was determined as 0.30±0.48 in Group I, 11.80±2.74 in Group II, 24.70±3.34 in Group III and 47.60±5.34 in Group IV. Similar studies have been carried out with other food additives and chemicals that support our findings. For example, Dönbak *et al.* (2002) investigated the cytogenetic effects of boric acid, which is used as a preservative additive in foods, on the *A. cepa* test material, resulting in boric acid causing the forma-

Table 2. Effects of different doses of benzyl benzoate on MN formation and Mitotic Index (MI).

Groups	MN Frequency Average±SD	Mitotic Index (MI)	Percent MI (%)
Group I	0.30±0.48^{d}	901.50±32.35^{a}	9.02
Group II	11.80±2.74^{c}	808.40±30.84^{b}	8.08
Group III	24.70±3.34^{b}	699.50±32.86^{c}	7.00
Group IV	47.60±5.34^{a}	534.70±16.67^{d}	5.35

*Group I: Control, Grup II: 10,000 mg/L benzyl benzoate, Grup III: 25,000 mg/L benzyl benzoate, Grup IV: 50,000 mg/L benzyl benzoate. For the determination of the mitotic index, 1,000 cells at each root tip in each group and 10,000 cells in total were analyzed. For the formation of MN, 100 cells each root tip in each group and were 1,000 cells in total were analyzed. Data were shown as mean ± standard deviation (SD) (n = 10). The statistical significance between the means was determined by using "one-way" ANOVA analysis of variance following the Duncan's test. The averages indicated by different letters in the same column were statistically significant (P<0.05).

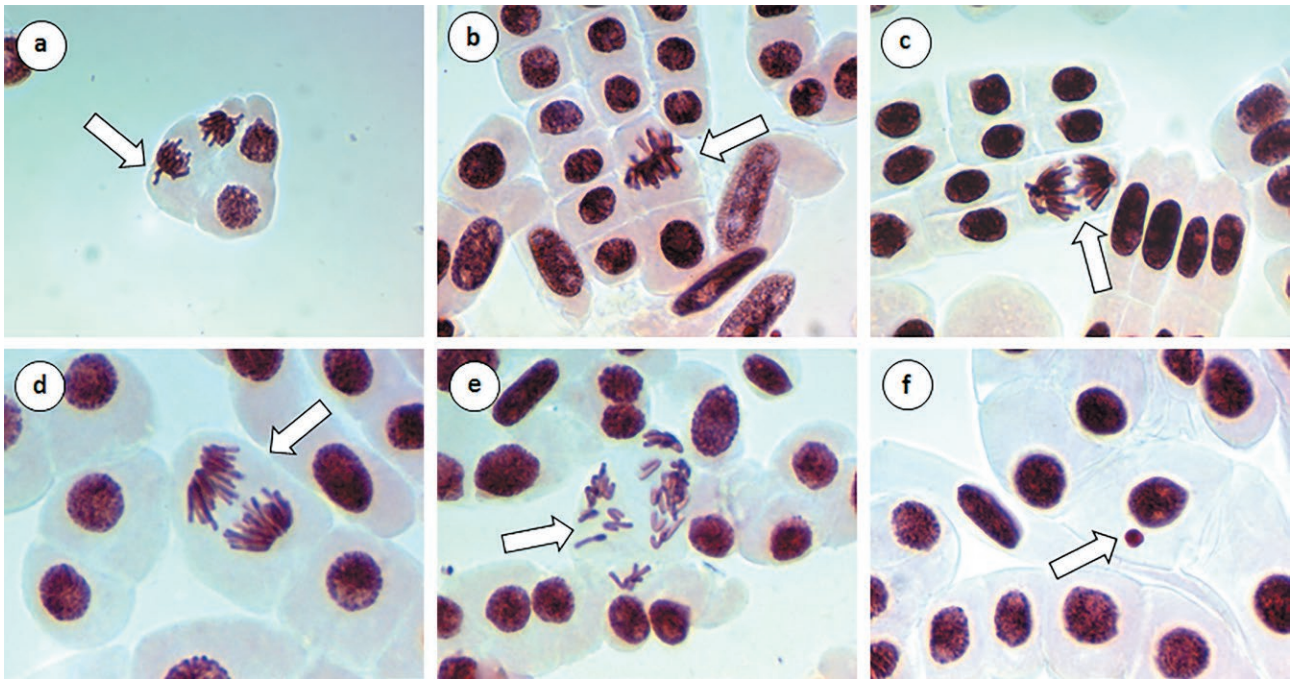

Figure 1. Chromosomal damages and formation of MN induced by benzyl benzoate (a: fragment, b: sticky chromosome, c: chromosome bridge, d: unequal distribution of chromatin dağılımı, e: c-mitosis, f: MN).

tion of MN. In another study, Gomes *et al.* (2013) investigated the cytogenetic effects of Bordeaux red (E-123), tartrazine yellow (E-102) and sunset yellow (E-110), which are used as colorant additives in foods, in *A. cepa* root tip cells. As a result, it has been reported that the application of these food additives causes the formation of MN.

The effects of benzyl benzoate on MN formation are shown in Table 3 and Figure 1. As a result of microscopic observations, it was determined that the frequency of chromosomal damages induced by benzyl benzoate application was fragment> sticky chromosome> chromosome bridge> unequal distribution of chromatin> c-mitosis. The highest effect of benzyl benzoate on chromosomes was determined as fragment formation. While no statistically significant chromosomal damages were observed in the control group (except several sticky chromosomes, chromosome bridge and c-mitosis), all chromosomal damages in the application groups increased due to the application dose and these increases were statistically significant ($P < 0.05$). Similar studies have been performed with other food additives and insecticides that support our findings. In a study conducted by Türkoğlu (2007), boric acid, sodium benzoate,

Table 3. Effects of different doses of benzyl benzoate on the frequency of chromosomal abnormalities

Groups	Number of root tips	Number of Mitotic Cells	FRG	SC	CB	UDC	CM
Group I	10	100	0.00 ± 0.00^{d}	0.30 ± 0.48^{d}	0.20 ± 0.42^{d}	0.00 ± 0.00^{d}	0.10 ± 0.32^{d}
Group II	10	100	17.10 ± 3.81^{c}	13.40 ± 2.91^{c}	10.80 ± 2.53^{c}	8.20 ± 2.20^{c}	3.60 ± 1.07^{c}
Group III	10	100	33.10 ± 4.77^{b}	27.60 ± 4.48^{b}	20.90 ± 5.15^{b}	14.80 ± 4.08^{b}	9.50 ± 2.07^{b}
Group IV	10	100	65.20 ± 7.36^{a}	46.70 ± 8.10^{a}	39.90 ± 8.12^{a}	30.20 ± 6.70^{a}	23.40 ± 5.62^{a}

*Group I: Control, Grup II: 10,000 mg/L benzyl benzoate, Grup III: 25,000 mg/L benzyl benzoate, Grup IV: 50,000 mg/L benzyl benzoate. Data were shown as mean ± standard deviation (SD) (n = 10). For chromosomal abnormalities, 100 cells at each root tip in each group and 1000 cells in total were analyzed. The statistical significance between the means was determined by using "one-way" ANOVA analysis of variance following the Duncan's test. The averages indicated by different letters in the same column were statistically significant ($P<0.05$). (FRG: fragment, SC: sticky chromosome, CB: chromosome bridge, UDC: unequal distribution of chromatin, CM: c-mitosis).

potassium citrate and citric acid used as preservatives in foods were applied to *A. cepa* bulbs in 20, 40, 60, 80 and 100 ppm doses, as a result, it was reported that chromosome damage occurred in anaphase bridge, c-mitosis, laggard chromosome and sticky chromosome, chromosome damages occurred due to the increase in application time and doses. In another study, Singh *et al.* (2007) treated *Hordeum vulgare* L. seeds with 0.01%, 0.1% and 0.5% concentrations of prophenophos insecticide and mancozeb fungicide. As a result, it was reported that chromosomal abnormalities occurred due to the application doses and chromosomal damages were reported as disturb metaphase, bridge, laggards, chromosomal breakage and diagonal anaphase.

In this study, it was determined that benzyl benzoate had an inhibitory effect on Mitotic Index (MI) in *A. cepa* root tip cells.

The effect of benzyl benzoate application on Mitotic Index (MI) in root cells of *A. cepa* is shown in Table 2. When the data were analyzed, MI was 9.02% in the control group, 10.85% in Group II, 7.00% in Group III and in Group IV it was determined as 5.35%. Depending on these findings, it was determined that MI was decreased with increasing doses of benzyl benzoate, in other words, the MI and benzyl benzoate administration dose showed inverse proportions. In addition, the differences between the groups were determined to be statistically significant ($P < 0.05$). Similar studies have been carried out with other food additives and insecticides that support our findings. Njagi and Gopalan (1982) applied different doses of sodium benzoate and sodium sulfite used as preservative additives in foods to the root tip cells of *Vicia faba* L., as a result, a reduction in MI was reported due to the administration dose. In another study, Rencüzoğulları *et al.* (2001), treated *A. cepa* test material with sodium metabisulphite used as a coloring additive in foods at doses of 7.5 mg/L, 15 mg/L and 30 mg/L for 10 and 20 hours, consequently, it was determined that MI decreased in both applications duration due to application doses.

As a result of microscopic observations, it was observed that the application of benzyl benzoate caused significant anatomic damages in *A. cepa* root tip cells. Anatomical examinations of root tips of the control and application groups application group are shown in Figures 2 and 3. These damages are in the form of necrosis, cell deformation, flattened cell nucleus, accumulation of some substances in cortex cells, cortex cell deformation, cortex cell wall thickening and unclear transmission tissue (Figure 2). As a result of the investigations, it was found that there was no anatomical change in the root

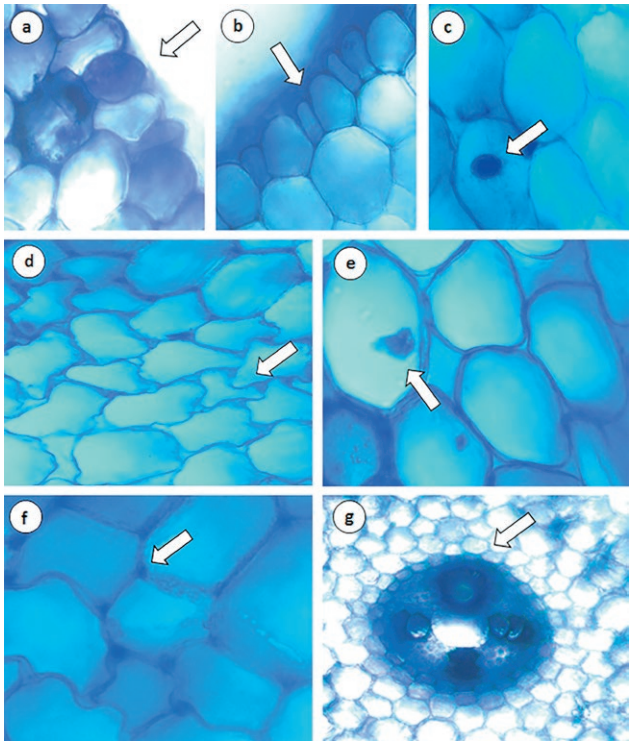

Figure 2. Anatomical changes induced by benzyl benzoate in root cell meristematic cells of *A.cepa* (a: necrosis, b: cell deformation, c: flattened cell nucleus, d: cortex cell deformation, e: accumulation of some substances in cortex cells, f: cortex cell wall thickening, g: unclear transmission tissue).

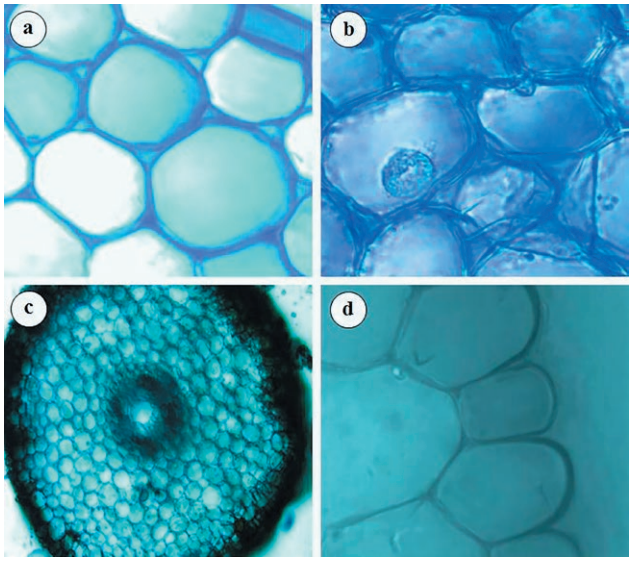

Figure 3. The appearance of the control group root tip meristematic cells (a: normal appearance of cortex cells, b: the usual shape of the cell nucleus, c: the usual appearance of the transmission tissue, d: normal appearance of epithelial cells).

tip cells and tissues of the control group (Figure 3), but an increase in anatomic damage rates was also observed in the application groups due to the increasing doses of benzyl benzoate. Since there is no comprehensive study on the anatomic damage caused by benzyl benzoate in plant root tip cells, the results are discussed with data from other chemical agents. Bıçakcı *et al.* (2017) reported that the application of diazinon cause anatomic damages in the root tip cells of *A. cepa* as unclear conduction tissue, flattened cell nucleus, cell deformation, thickening of cortex cell walls, necrosis and accumulation of some substances in the tissue of transmission, and the frequency of these damages increased due to application dose. Çavuşoğlu *et al.* (2011) reported that the application of glyphosate causes anatomic changes in the cell-root cell of *A. cepa*, unclear vascular tissue, cell deformation, unclear epidermis layer, binuclear cell and abnormal cell nucleus.

In our study, the effects of benzyl benzoate on lipid peroxidation in *A. cepa* root tip cells were also investigated. Lipid peroxidation is a metabolic process that causes oxidative degradation of reactive oxygen species (ROS) and lipids, especially polyunsaturated fatty acids and MDA production (Odjegba and Adeniran, 2015). MDA is an oxidative product of membrane lipids and a biological marker that indicates the level of oxidative stress (Janero, 1990). ROS damage the peroxidation of biological molecules including lipids, proteins, RNA and DNA (Shah *et al.*, 2001; Dinakar *et al.*, 2010). Free oxygen groups can act on DNA and cause mutations in nucleic acids and changes in chromosomes (Yarsan, 2014). The effects of benzyl benzoate application on root MDA levels are shown in Figure 4. When MDA levels were examined, the lowest MDA level was 10.00 µmol/g FW in the control group. SOD levels were found to be 1.45 times higher in Group II, 1.83 times higher in Group III and 2.21 times higher in Group IV compared to the control group. It was determined that the level of MDA increased with the increase in the benzyl benzoate doses. In addition, it was observed that the differences in the MDA level between the groups were statistically significant ($P < 0.05$). In the literature, because of the lack of a comprehensive study investigating the effect of benzyl benzoate on lipid peroxidation in plant test materials, our findings are discussed with similar studies examining the effects of other chemicals on lipid peroxidation in plants test materials. In the investigations chromium (IV) application of *A. cepa* root tip tissues (Patnaik *et al.* 2013), high-dose lead application on *Allium sativum* L. (Liu *et al.* 2009), bentazone herbicide application in rice (Wang *et al.* 2008) reported an increase in MDA levels in tissues according to applications.

Various defense mechanisms have been developed by aerobic organisms against free radicals. One of the so-called protective antioxidants CAT decomposes hydroperoxides or hydrogen peroxide and SOD reduces the formation of free radicals and active oxygen by quenching and modifying active oxygen (Comporti, 1993). The effects of benzyl benzoate application on root SOD and CAT activity are shown in Figure 5 and 6 respectively. The lowest SOD levels were measured as 75.00 U/mg FW in the control group. SOD levels in benzyl benzoate application groups were 94.00 U/mg in Group II, 157.00 U/mg in Group III and 180.70 U/mg in Group IV. It was determined that the level of SOD increased with the increase in benzyl benzoate dose. In addition, it was observed that the SOD level differences between the groups were statistically significant ($P < 0.05$). The level of SOD is thought to be increased as a result of ROS formation in the form of superoxide radi-

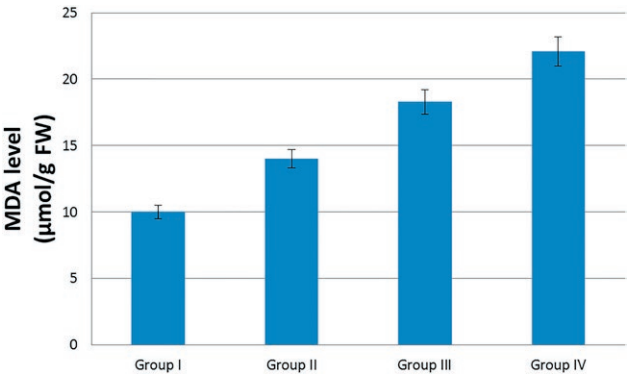

Figure 4. Effects of benzyl benzoate application on MDA levels. (Group I: Control, Grup II: 10,000 mg/L benzyl benzoate, Grup III: 25,000 mg/L benzyl benzoate, Grup IV: 50,000 mg/L benzyl benzoate). Vertical bars denote Standard Error (SE).

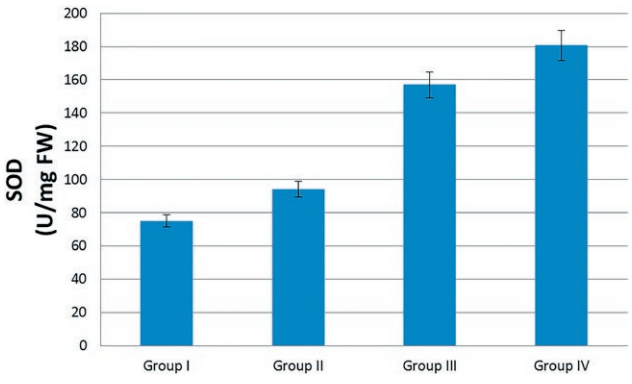

Figure 5. Effects of benzyl benzoate application on SOD activity (Group I: Control, Grup II: 10,000 mg/L benzyl benzoate, Grup III: 25,000 mg/L benzyl benzoate, Grup IV: 50,000 mg/L benzyl benzoate). Vertical bars denote Standard Error (SE).

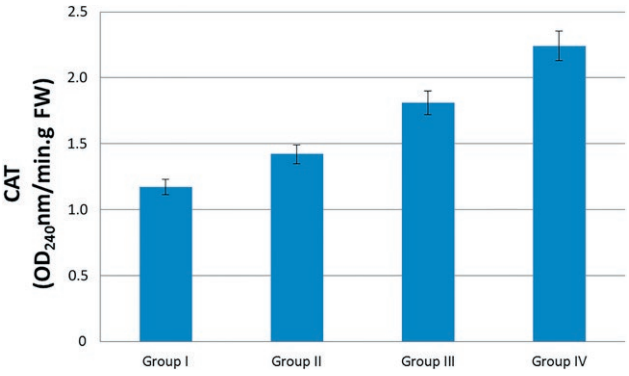

Figure 6. Effects of benzyl benzoate application on CAT activity (Group I: Control, Grup II: 10,000 mg/L benzyl benzoate, Grup III: 25,000 mg/L benzyl benzoate, Grup IV: 50,000 mg/L benzyl benzoate). Vertical bars denote Standard Error (SE).

cals by exposure to benzyl benzoate. Superoxide is a key component of signal transduction triggers genes responsible for antioxidant enzymes, including SOD (Alavarez and Lamb, 1997). The lowest CAT levels were measured as 1.17 OD_{240nm}/min.g FW in the control group. CAT levels in benzyl benzoate application groups were 1.42 OD_{240nm}/min.g FW in Group II, 1.81 OD_{240nm}/min.g FW in Group III and 2.24 OD_{240nm}/min.g FW in Group IV. Increased levels of SOD and CAT were determined by increasing benzyl benzoate dose. Similarly, in many studies, antioxidant enzyme activities have been reported to increase in *Allium* species due to stress-induced by other chemicals. Application of different doses of chlorpyrifos and mancozeb insecticides caused an increase in CAT and SOD levels in *A. cepa* leaves (Fatma *et al.* 2018), the increase in SOD and CAT levels due to the application doses and duration of cypermethrin insecticide on *A. cepa* root tips (Çavuşoğlu *et al.* 2012a), the application of cadmium inhibited SOD and CAT levels in *Allium sativum* L. leaves but when the application was continued the increase in SOD and CAT levels was reported (Zhang *et al.* 2005).

CONCLUSIONS

When all the data obtained in the study were examined; benzyl benzoate application showed inhibitory effects on physiological parameters investigated in *A. cepa* test material. This is thought to be due to the inhibitory effect of benzyl benzoate on the cell cycle. Inhibition in physiological parameters is considered as an indicator of benzoate toxicity. Benzyl benzoate increases the occurrence of chromosomal damage with the frequency of MN and decreases in the rate of MI suggests that it may be caused by the production of more ROS that can be detoxified by the cellular defense mechanisms and by causing the damage to DNA by being tolerated. Increases in MDA, SOD and CAT levels also support this situation. In addition, due to the application of benzyl benzoate, the anatomical changes occurring in the transmission tissue and root tip cells may be due to the defense mechanisms developed to inhibit the cellular uptake of the benzyl benzoate developed by the plant. Despite these mechanisms, high benzyl benzoate doses may be caused the penetration of the substance into the plant.

As a result; benzyl benzoate, which is used in many different fields, such as food, health, cosmetics and agricultural production, has been identified using *A. cepa* test material, which can show toxic effects if it reaches certain concentrations. For this reason, the use of benzyl benzoate exposure should be avoided considering the damages that can be caused to living things. In cases such as disease treatments where the use is essential, the appropriate dosage and duration range should be determined and the areas of use should be limited.

In this study conducted using *A. cepa* test material, it was found that physiological parameters such as germination percentage, root length and weight gain are important precursors in the rapid detection of toxicity. Cytogenetic parameters such as chromosomal abnormalities, MN formation and MI ratio are sensitive biomarkers in the biological monitoring of toxicity. In addition, it was determined that the determination of MDA, SOD and CAT levels contributed to explaining the causes and effect mechanisms of toxicity, the anatomical changes occurring in the root tip meristematic cells were found to contribute to the understanding of the cellular responses of the plant during the incorporation of the chemical agent into the plant.

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Karyological variability and chromosomal asymmetry in highland cultivars of *Chenopodium quinoa* Willd. (Amaranthaceae)

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Abstract. *Chenopodium quinoa* Willd. is rapidly gaining importance worldwide as a superfood. However, structural diversity and asymmetry analyses of chromosomes of different cultivars of the species are largely understudied primarily owing to their small chromosomes. In this paper, karyomorphological investigations were performed on 21 cultivars of *C. quinoa* with varying seed morphology cultivated widely in the highland regions of the Andes, which is the center of domestication of this species. Somatic chromosome number was found to be 2n= 36 in all cultivars with no occurrence of mixoploidy. Lengths of individual chromosomes varied between 0.63–6.53 µm, with their short arms ranging from 0.25–2.95 µm and long arms between 0.38–3.58 µm. Types of primary constriction ranged from median to sub–terminal. One pair of chromosome in each complement possessed a secondary constriction. Chromosome complements of all cultivars belonged to the asymmetry class 2B with an average asymmetry index value of 3.21±0.61. Values of intra- and inter-chromosomal asymmetry indices were 0.30±0.02 and 0.20±0.02 respectively across all cultivars. The average coefficients of variation of chromosome lengths was 19.77±2.11 and average centromeric index was 16.28±2.12. Arm ratio of the chromosomes varied from 0.34 to 5.76. The mean values of karyotypic asymmetry, symmetry index and karyotype asymmetry index percentage were 17.69±1.53, 147.69±5.54 and 58.71±0.86 respectively. Pearson correlation revealed strong correlation within inter– and intra–chromosomal asymmetry indices. Our analyses uncovered higher chromosomal variation in quinoa than previously found with high inter–varietal similarities among the studied cultivars, revealed from scattered diagrams between asymmetry indices.

Keywords: *Chenopodium quinoa*, chromosome, karyotype**,** chromosomal asymmetry, inter-varietal symmetry.

INTRODUCTION

Sustainable agricultural systems generally focus on traditional commercial food crops, which might not be adequate in the near future to fulfill the projected increase in global consumption due to increase in population. This, along with rapid climate change, has necessitated the investigation and

exploitation of alternative crop plants to complement the projected deficit (Graf *et al.* 2015). Recent research has focused on the South American crop *Chenopodium quinoa* (quinoa), which the Food and Agriculture Organization of the United Nations have touted as a crop that can mitigate global food demand to a large extent (Graf *et al.* 2015). This plant's adaptability to extreme agro–ecological conditions together with its balanced proportion of amino acids, carbohydrates, lipids, vitamins and minerals, low glycemic acid content and its gluten–free nature, have resulted in expansion of its cultivation outside its native region (Ruiz *et al.* 2014; Pereira *et al.* 2019). Improvement programs of the crop include reduction in saponin content of its seed coat, increasing compactness of its inflorescence, high stress tolerance and resistance to pests and pathogens (Murphy *et al.* 2016; Jarvis *et al.* 2017). Success of these programs demand identification of existing genetic divergence in the species.

An initial idea of genetic variability among plants can be perceived by analyzing cytological characters (Guerra 2008). Most crop plants have undergone recent polyploidization events (Renny-Byfield and Wendel 2014). Polyploidy results in variable chromosome numbers and the ensuing chromosomal changes like translocations and inversions give rise to structural variabilities (Leitch and Leitch 2008). Analyses and comparison of these 2 parameters, viz. chromosome number and structure, among related taxa is considered to be a reliable approach for quantitative interpretation of their similarity or divergence among related plants (Bennett and Leitch 2005). Generally considered an allotetraploid with a basic chromosome number x= 9 (Krak *et al.* 2016; Mandák *et al.* 2016), the reported somatic chromosome counts of quinoa predominantly showed an invariable 2n= 36 (Kjellmark 1934; Wulff 1936; Cárdenas and Hawkes 1948; Heiser 1963; Gandarillas and Luizaga 1967; Giusti 1970; Kolano *et al.* 2001, 2012; Rahiminejad and Gornall 2004; Bhargava *et al.* 2006; Palomino *et al.* 2008; Mandák *et al.* 2016). However, there are limited studies on variations in chromosome structure and asymmetry in the species mainly due to their small size, which makes common karyotyping of the species difficult (Kolano *et al.* 2001, 2012). Such studies, when present, were mostly limited to examining only 1 or 2 varieties restricting the possibility of understanding inter-varietal chromosomal differences, or, were based on assessments of insufficient asymmetry parameters (Kolano *et al.* 2001, 2012; Bhargava *et al.* 2006; Maughan *et al.* 2006; Palomino *et al.* 2008; Yangquanwei *et al.* 2013).

Earliest reports of domestication and cultivation of quinoa can be traced back to about 7,000 years ago to the Inca dynasty in South American Andes, especially near Lake Titicaca from Peru and Bolivia that constituted the ancestral highland ecotype of this species (Jarvis *et al.* 2017). Although variations in prominent morphological markers for commercial quinoa like seed size and colour, exist within the cultivated varieties of South America, it can be argued that, prolonged farming of a species at and around its center of domestication and diversity might expose them to the risk of genetic erosion, resulting in low genetic variability (Gonzalo *et al.* 2019). Existence of such genetic erosion, undesirable for germplasm improvement and conservation programmes, might be realized from cytological studies; however, chromosomal study of quinoa from this region is not forthcoming.

The objectives of our work were (1) to analyze the karyotype structure of highland varieties of quinoa, (2) to determine the extent of karyotype differentiation among the varieties, (3) to evaluate chromosomal asymmetry in the varieties. We investigated 21 morphologically variable highland cultivars of quinoa, and, performed karyotypic analyses based on asymmetry parameters that could help in elucidating worthwhile information on the inter–varietal divergence of quinoa.

MATERIALS AND METHODS

Materials

For the purpose of this study, seeds of white, yellow, red and black–coloured varieties were selected from the highland ecotypes of quinoa, their sizes ranging from 1.5 to 3.0 cm in diameter. These cultivars were collected from Peru and Bolivia around Lake Titicaca (Figure 1). Seeds from at least 5 individuals of each cultivar were germinated on moist filter paper in Petri plates, kept in dark for 24–48 h at room temperature for cytological study. Growing root tips of freshly germinated seedlings were used for cytological analyses.

Methods

Actively growing 1.5–2 cm long root tips of seedlings were harvested after 1 to 2 days of germination initiation, between 10 AM to 11 AM, for mitotic study. Pretreatment of the harvested root tips were performed in aqueous solution of 2 mM 8–hydroxyquinoline for 3 hours at 10°C after which the root tips were fixed overnight in Carnoy's fixative at 10°C. Hydrolysis, staining of the root tips and slide preparation were done following the protocol of Sun *et al.* (2017). Squashed root tips were observed under Olympus BX51 microscope (Olympus, Germany) at a magnification of 1000X. Well scattered

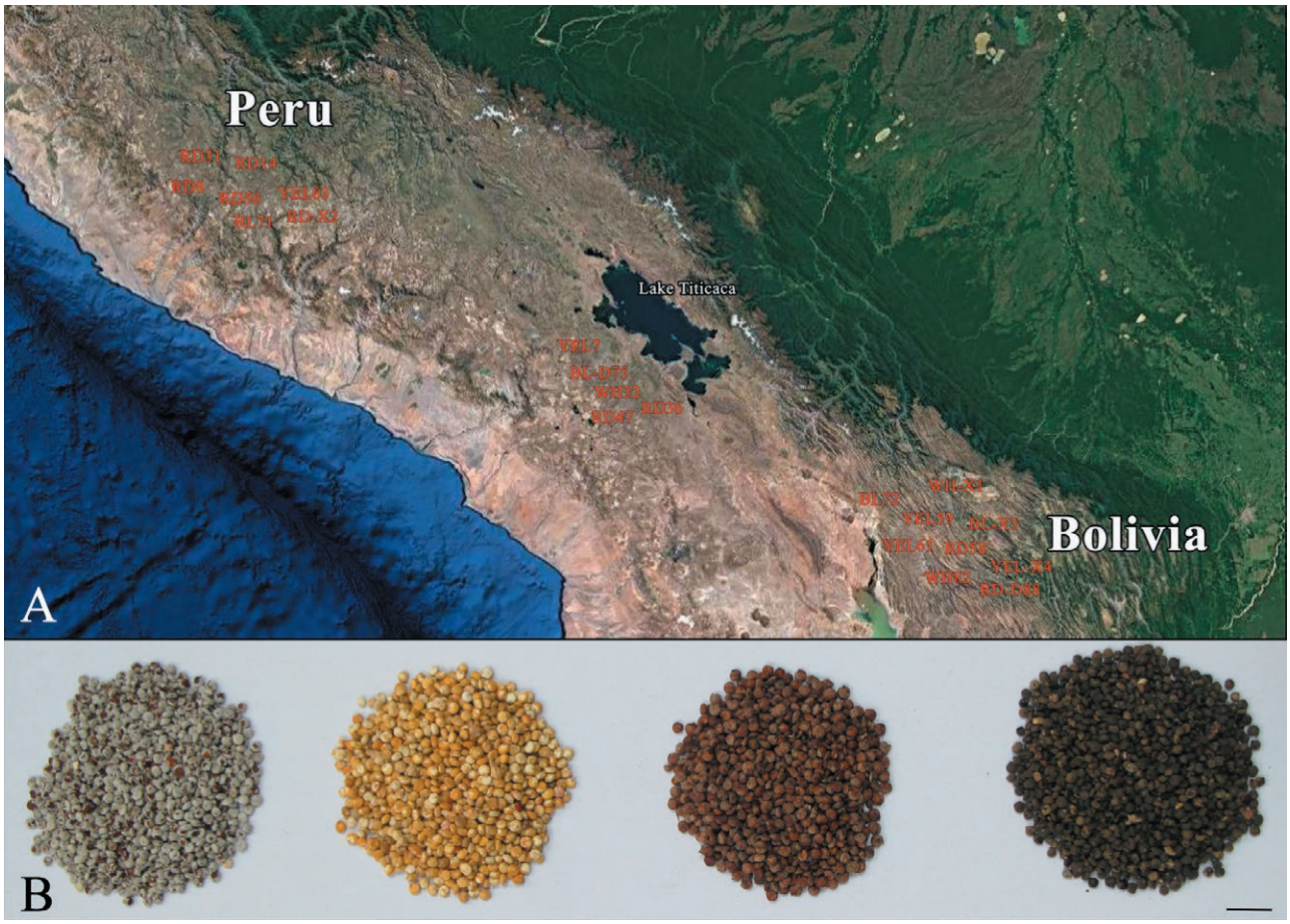

Figure 1. (A) Map showing the collection area of the studied cultivars (B) Photographs of seeds showing morphological variation (bar=1 cm).

metaphase plates were photographed with a mounted digital camera using the software ScopePhoto (ver. 3.0.12.444) (ScopeTek, Hangzhou, China).

Chromosome numbers were counted from at least 50 metaphase plates prepared from 50 different seedlings for each cultivar of quinoa to ensure the uniformity of somatic chromosome numbers in all the plants. Among them, at least 30 best metaphase plates were selected for karyotype analyses with a minimum of 10 seedlings chosen per cultivar.

Lengths of chromosome arms were measured from at least 5 best metaphase plates for each cultivar using KaryoType v2 software (Altınordu *et al.* 2016). The lengths of chromosomes and respective centromeric indices of each metaphase plate, obtained from this data, were used to describe the chromosome morphology following the nomenclatural system of Levan *et al.* (1964), and, to prepare their idiograms with OriginPro 2018 software (OriginLab Corporation, USA). Asymmetry of karyotypes of each cultivar were calculated using Stebbin's classification (1971) and AI (karyotype asymmetry index), intra-chromosomal asymmetry indices using A1 (intra-chromosomal asymmetry index), CV_{CI} (coefficient of variation of centromeric index), M_{CA} (mean centromeric asymmetry), Syi (symmetric index), AsK% (Karyotype asymmetry index percentage) and AR (arm ratio), and inter-chromosomal asymmetry using A2 (inter-chromosomal asymmetry index), CV_{CL} (coefficient of variation of chromosome length) (for details see Bhadra and Bandyopadhyay 2015). Pearson correlation among these intra- and inter-chromosomal asymmetry parameters was calculated in SPSS (version 21) software (München, Germany).

RESULTS

The chromosomes of studied cultivars of *Chenopodium quinoa* were small, though clearly visible with conspicuous centromeric regions.

Table 1. Chromosome numbers, karyotype formulae, morphometric parameters and values of asymmetry indices studies in 21 *Chenopodium quinoa* varieties.

Local variety name	2n	Karyotype formula	Short arm range (average) in μm	Long arm range (average) in μm	Total length range (average) in μm
BL71	36	2M+22m+8Sm+2St+2m:Sm	0.42–1.77 (1.03±0.25)	0.80–2.64 (1.49±0.34)	1.35–4.24 (2.56±0.50)
BL72	36	8M+14m+10m+2St+2m:Sm	0.42–1.92 (1.04±0.31)	0.50–3.02 (1.52±0.39)	0.92–4.19 (2.59±0.59)
BL–D75	36	18m+14Sm+2St+2M:Sm	0.39–2.15 (1.04±0.26)	0.46–2.83 (1.55±0.40)	0.85–4.51 (2.62±0.57)
BL–X3	36	6M+8m+16Sm+4St+2m:Sm	0.59–2.19 (1.21±0.28)	1.04–3.45 (1.91±0.36)	1.88–4.61 (3.16±0.42)
RD14	36	6M+14m+12Sm+2St+2m:Sm	0.52–1.79 (1.10±0.25)	0.65–2.88 (1.69±0.44)	1.17–3.96 (2.82±0.50)
RD31	36	4M+18m+10Sm+2St+2M:Sm	0.44–2.95 (1.13±0.33)	0.55–3.58 (1.71±0.42)	0.99–6.53 (2.88±0.66)
RD36	36	4M+26m+4Sm+2m:Sm	0.40–1.34 (0.95±0.18)	0.57–2.12 (1.30±0.23)	0.97–3.95 (2.28±0.38)
RD47	36	4M+20m+8Sm+2St+2m:Sm	0.40–1.83 (1.16±0.26)	0.46–2.82 (1.59±0.30)	0.86–4.41 (2.78±0.49)
RD56	36	8M+20m+6Sm+2M:Sm	0.37–1.48 (0.99±0.24)	0.49–2.09 (1.41±0.29)	0.86–3.85 (2.44±0.48)
RD58	36	8M+16m+8Sm+2St+2M:Sm	0.37–2.00 (1.13±0.37)	0.52–3.00 (1.60±0.44)	0.89–4.82 (2.76± 0.70)
RD8	36	2M+16m+12Sm+4St+2m:Sm	0.33–1.93 (1.10±0.30)	0.60–3.66 (1.67±0.44)	0.93–5.45 (2.81±0.64)
RD–D88	36	6M+16m+10Sm+2St+2m:Sm	0.42–1.85 (1.10±0.32)	0.50–2.96 (1.61± 0.35)	0.92–4.44 (2.75±0.58)
RD–X2	36	2M+12m+18Sm+2St+2m:Sm	0.25–1.64 (1.00±0.24)	0.38–2.38 (1.52±0.30)	0.63–3.94 (2.55±0.47)
WH32	36	6M+20m+6Sm+2St+2m:Sm	0.68–2.57 (1.18±0.32)	0.54–2.87 (1.77±0.40)	1.03–5.44 (2.99±0.58)
WH82	36	8M+22m+4Sm+2m:Sm	0.48–1.81 (1.10±0.26)	0.58–2.52 (1.49±0.32)	1.06–4.18 (2.62±0.54)
WH–X1	36	2M+18m+12Sm+2St+2m:Sm	0.35–1.42 (0.85±0.20)	0.38–2.42 (1.26±0.26)	0.73–3.20 (2.14±0.40)
YEL59	36	8M+18m+6Sm+2St+2M:Sm	0.49–1.97 (1.16±0.28)	0.73–2.83 (1.63±0.36)	1.22–4.34 (2.82±0.54)
YEL61	36	4M+16m+12Sm+2St+2m:Sm	0.42–1.66 (0.97±0.26)	0.47–2.91 (1.54±0.35)	0.92–3.98 (2.55±0.50)
YEL63	36	6M+22m+4Sm+2St+2m:Sm	0.47–1.75 (1.06±0.29)	0.59–2.81 (1.59±0.42)	1.06–4.36 (2.69±0.59)
YEL7	36	2M+22m+10Sm+2m:Sm	0.44–1.82 (1.16±0.26)	0.53–2.79 (1.70±0.36)	0.97–5.02 (2.92±0.54)
YEL–X4	36	4M+24m+6Sm+2m:Sm	0.53–1.68 (1.07±0.24)	0.77–2.61 (1.63±0.31)	1.35–4.50 (2.74±0.42)

Local variety name	Centromeric index range (average) (i)	Karyotype asymmetry class	Arm ratio range (average) (AR)	Value of relative chromatin (VRC)	Total form percentage (TF%)	Relative length percentage range (RL)
BL71	18.79–49.76 (41.17±6.83)	2B	0.70–4.32 (1.51±0.06)	5.11±0.61	41.78±0.96	1.56–4.56
BL72	16.35–49.76 (40.52±7.72)	2B	0.69–5.12 (1.57±0.11)	5.19±0.64	41.35±1.06	0.86–4.06
BL–D75	19.43–49.70 (40.52±6.37)	2B	0.57–4.14 (1.54±0.13)	5.24±0.99	40.80±2.13	0.93–4.46
BL–X3	21.69–49.82 (39.40±7.63)	2B	0.41–3.61 (1.65±0.22)	6.32±0.88	39.76±3.18	1.91–3.64
RD14	14.79–50 (40.01±8.03)	2B	0.54–5.76 (1.62±0.11)	5.65±0.13	40.21±0.36	1.63–3.95
RD31	22.41–49.78 (40.13±6.44)	2B	0.61–3.46 (1.59±0.06)	5.76±1.52	40.33±0.93	1.20–6.74
RD36	31.22–49.76 (42.48±4.17)	2B	0.60–2.20 (1.38±0.02)	4.56±0.47	42.98±0.27	1.26–4.30
RD47	24.31–49.84 (42.36±5.77)	2B	0.77–3.11 (1.43±0.13)	5.56±0.56	42.77±1.88	0.96–4.02
RD56	25.73–49.84 (41.84±5.65)	2B	0.60–2.88 (1.46±0.09)	4.87±0.22	42.26±1.37	0.95–4.29
RD58	15.56–49.81 (41.44±7.46)	2B	0.68–5.42 (1.52±0.10)	5.53±0.89	42.08±0.96	0.89–4.57
RD8	18.16–49.32 (40.02±6.75)	2B	0.55–4.51 (1.59±0.21)	5.63±0.46	40.52±2.49	0.97–4.92
RD–D88	21.32–49.82 (40.35±7.40)	2B	0.39–3.69 (1.57±0.12)	5.49±0.81	41.34±1.44	1.10–5.26
RD–X2	20.92–50 (39.99±6.66)	2B	0.54–3.78 (1.59±0.28)	5.10±0.06	40.48±4.02	0.68–4.24
WH32	19.82–49.64 (40.52±7.08)	2B	0.53–4.04 (1.58±0.09)	5.98±0.45	40.63±1.43	0.88–4.65
WH82	25.00–49.72 (42.38±5.24)	2B	0.70–3.00 (1.38±0.04)	5.25±0.72	43.23±0.71	1.06–4.07
WH–X1	24.37–50 (40.97±5.53)	2B	0.44–3.10 (1.51± 0.20)	4.29±0.37	41.30±2.96	1.01–3.88
YEL59	22.09–49.77 (41.92±6.15)	2B	0.76–3.53 (1.46±0.10)	5.64±0.88	42.38±1.52	1.29–3.99
YEL61	18.03–49.69 (39.05±7.23)	2B	0.34–4.55 (1.66±0.16)	5.09±0.59	39.69±1.53	1.04–4.49
YEL63	17.07–49.83 (40.57±7.38)	2B	0.41–4.86 (1.56±0.10)	5.39±0.53	40.99±2.15	1.04–4.26
YEL7	25.69–49.60 (40.97±6.96)	2B	0.67–3.28 (1.51±0.05)	5.84±0.46	41.69±0.48	0.85–4.41
YEL–X4	18.03–50 (39.87±6.91)	2B	0.35–4.55 (1.62±0.41)	5.47±0.28	40.49±4.74	1.35–4.51

Local variety name	Difference of relative length (DRL)	Intra–chromosomal asymmetry index (A1)	Inter–chromosomal asymmetry index (A2)	Coefficient of variation of chromosome length (CV_{CL})	Coefficient of variation of centromeric index (CV_{CI})
BL71	2.52±0.54	0.29±0.02	0.19±0.03	19.46±2.92	16.61±1.14
BL72	2.72±0.30	0.29±0.03	0.22±0.03	22.48±2.72	18.61±1.68
BL–D75	2.92±0.15	0.31±0.06	0.22±0.04	22.35±3.63	15.79±1.83
BL–X3	1.49±0.30	0.33±0.07	0.14±0.03	13.71±3.18	19.58±4.55
RD14	2.18±0.06	0.31±0.01	0.18±0.03	17.65±2.80	20.08±3.43
RD31	4.65±0.95	0.33±0.02	0.23±0.05	23.28±4.90	16.04±2.22
RD36	2.54±0.30	0.26±0.01	0.17±0.01	16.75±1.13	9.80±0.38
RD47	2.48±0.18	0.26±0.05	0.18±0.01	17.78±0.12	13.66±2.14
RD56	2.62±0.38	0.28±0.03	0.20±0.01	19.76±0.73	13.56±3.10
RD58	3.07±0.14	0.27±0.01	0.25±0.02	25.17±2.28	18.04±4.42
RD8	3.06±0.34	0.32±0.06	0.23±0.02	22.78±2.36	17.10±4.25
RD–D88	3.11±1.01	0.30±0.04	0.21±0.05	21.48±5.02	18.46±3.91
RD–X2	2.90±0.59	0.32±0.10	0.18±0.03	18.42±2.72	16.81±2.54
WH32	2.73±0.74	0.31±0.04	0.20±0.03	19.47±2.67	17.54±2.46
WH82	2.56±0.27	0.25±0.02	0.20±0.03	20.35±3.37	12.35±0.94
WH–X1	2.29±0.46	0.30±0.08	0.19±0.02	18.78±1.74	13.68±3.97
YEL59	2.34±0.27	0.27±0.03	0.19±0.02	19.44±2.22	14.69±3.76
YEL61	2.75±0.95	0.34±0.03	0.20±0.05	20.14±4.61	18.63±5.77
YEL63	2.53±0.70	0.30±0.05	0.22±0.04	22.05±4.30	18.24±2.49
YEL7	2.89±0.60	0.29±0.02	0.18±0.02	18.32±2.18	14.65±0.44
YEL–X4	2.26±0.80	0.32±0.12	0.15±0.03	15.52±3.59	17.93±7.93

Local variety name	Karyotype asymmetry index (AI)	Mean centromeric asymmetry (M_{CA})	Symmetric index (Syi)	Karyotype asymmetry index percentage (AsK%)
BL71	3.21±0.24	16.80±1.39	144.12±5.02	58.22±0.96
BL72	4.20±0.83	17.47±2.05	146.47±6.20	58.65±1.06
BL–D75	3.51±0.51	18.30±3.74	150.64±14.17	59.20±2.13
BL–X3	2.59±0.23	20.43±5.43	157.21±20.92	60.24±3.18
RD14	3.48±0.18	19.08±0.69	154.02±2.09	59.79±0.36
RD31	3.66±0.27	19.46±1.48	153.07±5.90	59.67±0.93
RD36	1.64±0.05	14.55±0.73	136.77±2.15	57.02±0.27
RD47	2.43±0.39	15.04±3.37	138.12±10.76	57.23±1.88
RD56	2.69±0.70	16.18±2.37	142.12±7.60	57.73±1.37
RD58	4.61±1.47	16.34±1.52	141.87±5.68	57.92±0.96
RD8	3.91±1.15	19.02±4.76	152.55±17.95	59.48±2.49
RD–D88	3.97±1.37	18.32±3.14	147.19±9.70	58.66±1.44
RD–X2	3.14±0.94	19.26±7.26	153.63±27.35	59.52±4.02
WH32	3.41±0.60	18.70±2.72	150.94±9.46	59.37±1.43
WH82	2.53±0.57	14.24±1.45	135.69±3.16	56.77±0.71
WH–X1	2.53±0.54	17.70±5.75	147.95±18.65	58.70±2.96
YEL59	2.81±0.45	15.52±2.37	140.52±9.14	57.62±1.52
YEL61	3.57±0.18	20.85±2.73	158.16±9.74	60.31±1.53
YEL63	4.09±1.36	17.98±3.48	149.65±12.98	59.01±2.15
YEL7	2.68±0.27	17.03±1.24	145.88±4.32	58.31±0.48
YEL–X4	2.66±0.84	19.28±8.95	154.89±32.99	59.51±4.74

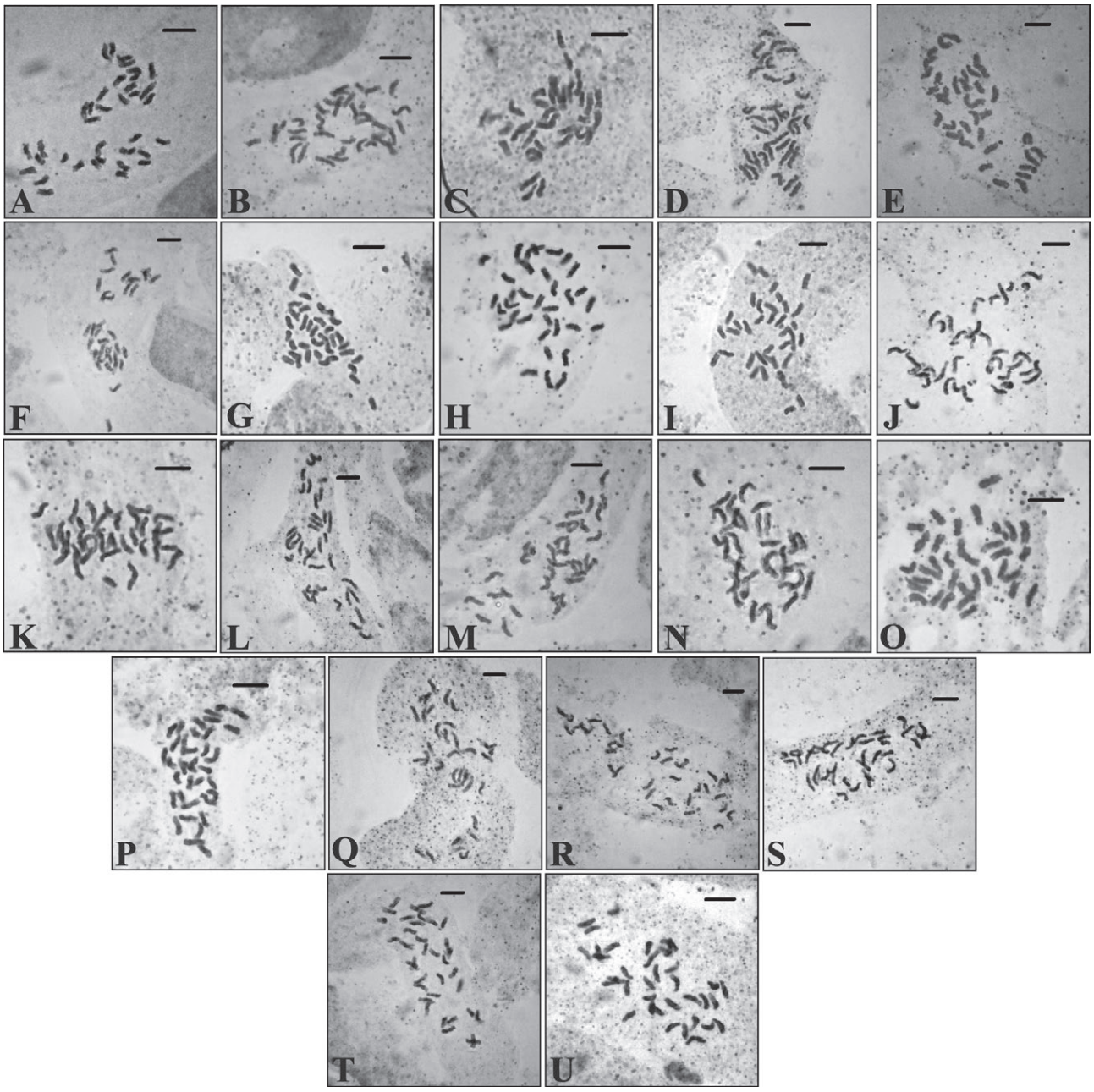

Figure 2. Somatic metaphase chromosomes of *Chenopodium quinoa* (2n=36): (A) BL71 (B) BL72 (C) BL–D75 (D) BL–X3 (E) RD14 (F) RD31 (G) RD36 (H) RD47 (I) RD56 (J) RD58 (K) RD8 (L) RD–D88 (M) RD–X2 (N) WH32 (O) WH82 (P) WH–X1 (Q) YEL59 (R) YEL61 (S) YEL63 (T) YEL71 (U) YEL–X4 (bar= 5 μm).

Somatic cells of all the cultivars exhibited a uniform chromosome number of 2n= 36 (Table 1, Figure 2). Lengths of chromosomes in all the cultivars varied from 0.63 μm to 6.53 μm, with the highest average chromosome length being present in the cultivar BL–X3 and the lowest in WH–X1 (Figure 3 A–F). Short arms were 0.25 μm to 2.95 μm in length, while long arms showed a variation of 0.38 μm to 3.66 μm. Centromeric indices ranged from 50% to 14.79% resulting in primary constrictions varying from median to sub-terminal types, though one cultivar did not possess any chromosome with median constriction and chromosomes of five cultivars did not exhibit sub-terminal constrictions. Number of chromosomes with each type of primary constriction also var-

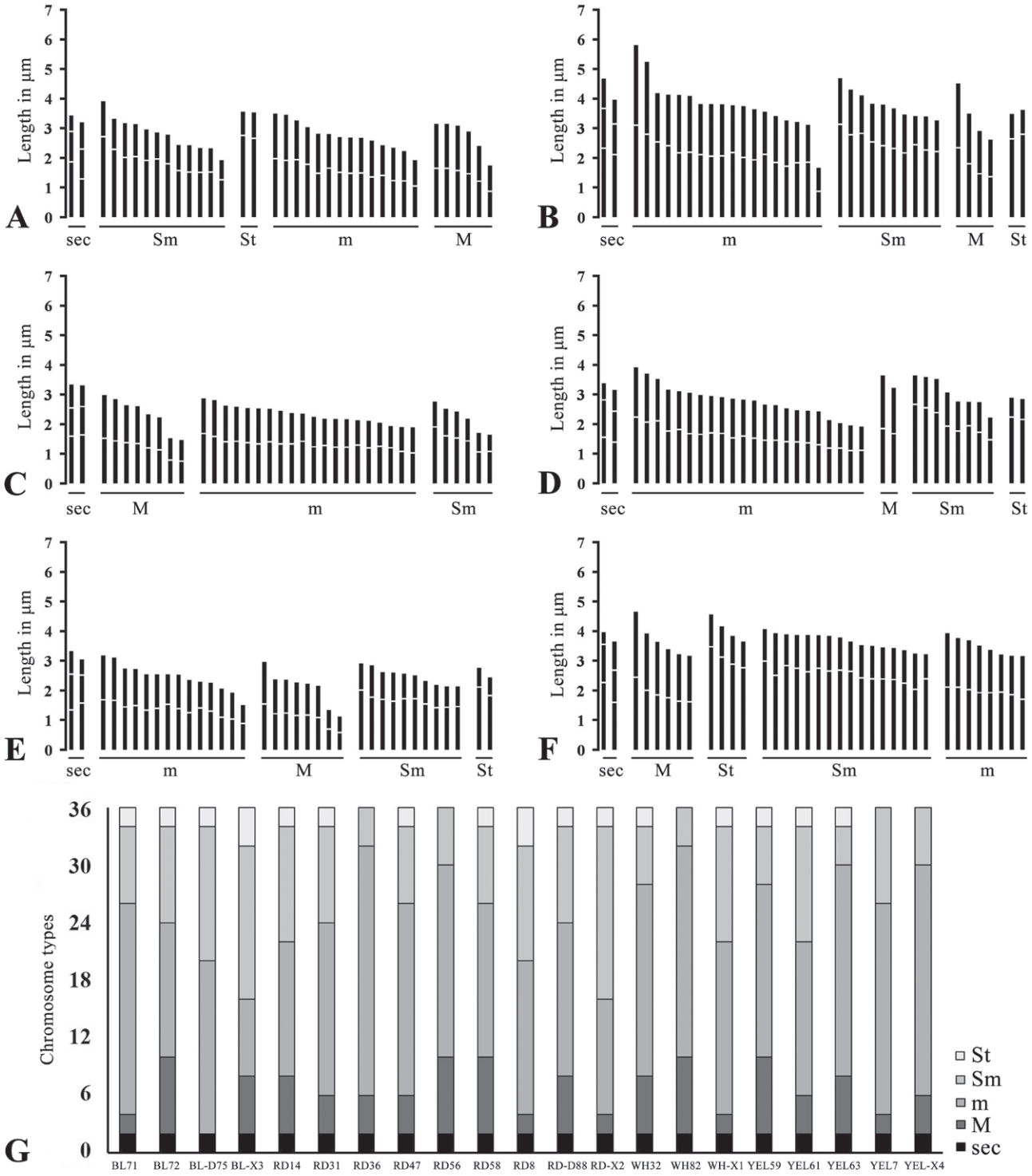

Figure 3. Idiograms of some cultivars of *Chenopodium quinoa*: (A) RD14 (B) RD31 (C) RD56 (D) BL71 (E) BL72 (F) BL–X3 (G) Comparative graphical representation of distribution of constriction types in the cultivars of *Chenopodium quinoa* (M= median constriction, m= nearly median constriction, Sm= sub–median constriction, St= sub–terminal constriction, sec= secondary constriction).

ied among the cultivars. All of the cultivars revealed 2 chromosomes possessing secondary constrictions, with one constriction in median or nearly median region and the other in sub–median region (Figure 3 G).

The inter- and intra-chromosomal asymmetry indices were calculated on the basis of chromosome lengths and centromeric indices. Stebbins classification (1971) placed the karyotype of quinoa in 2B category while AI ranged from 1.64 in RD36 to 4.61 in RD58. The complementary indices A1 and A2 ranged from 0.25 to 0.34, and, 0.15 to 0.25 respectively. Values of CV_{CL} and CV_{CI} varied from 15.52–25.17, and, 9.80–20.08 respectively, while that of M_{CA} was 14.24–20.85. Values of average AR ranged from 1.38 to 1.66, with the lowest recorded in YEL61 and highest in RD14. The values of Syi and Ask% ranged from 135.69 to 158.16, and, 56.77% to 60.31% respectively.

DISCUSSION

Domestication of plants has been impacted by polyploidy since the beginning of agriculture, with approximately 40–70% of cultivated plants exhibiting polyploidy (Hilu 1993, Sattler *et al.* 2016). Polyploids, especially allopolyploids show hybrid vigour with increased growth rates and higher productivity that favours their artificial selection over their diploid progenitors (Renny–Byfield and Wendel 2014). Considering Goldblatt's (1980) assumption of polyploid determination, quinoa with the basic chromosome number of x= 9, is a tetraploid with 2n= 36 somatic chromosomes, a condition similar to the closely related species *C. hircinum* and *C. berlandieri* (Fuentes–Bazan *et al.* 2012; Mandák *et al.* 2016; Jarvis *et al.* 2017). Studies have endorsed its allotetraploid nature, hypothesizing possible hybridization between a North American and a Eurasian diploid species, whose identities are yet unknown (Ward 2000; Jarvis *et al.* 2017). However, polyploid establishment and propagation is often hindered by irregular meiotic segregation of chromosomes that lead to chromosomal abnormality and reproductive sterility (Renny–Byfield and Wendel 2014). On the contrary, stability in the number of somatic chromosomes of this species, especially in those cutivars that were reported from Bolivia, Chile and Peru (Cárdenas and Hawkes 1948; Gandarillas and Luizaga 1967; Kolano *et al.* 2012), and the apparent absence of evidences of mixoploidy in the highland varieties investigated in the present study, both failed to give credence to sporadic reports of mixoploidy (Kawatani and Ohno 1950; Gandarillas 1979; Wang *et al.* 1993). This could in effect perhaps lead to the high fertility of quinoa (Ward 2000) that, along with its self–pollinating nature, has facilitated its extensive propagation, especially in new regions.

Karyomorphological trait variations resulting from structural changes of chromosomes, primarily as a result of rearrangement of chromosomal parts including translocation, inversion and/or deletion, is an important character for understanding relationship among closely related taxa (Peruzzi and Altinordu 2014). However, no published record of structural details of somatic chromosomes of quinoa was available until the beginning of this century. Present study recorded small somatic chromosomes with higher length variations, between 0.63–6.53 µm, and the longest chromosome was almost double the length of previously recorded longest chromosome which was 3.30 µm (Kolano *et al.* 2001; Palomino *et al.* 2008; Yangquanwei *et al.* 2013). This difference can be attributed to (a) the variable cytological techniques used in these studies that affect the degree of chromosomal condensation (Palomino *et al.* 2008), and, (b) the use of only 1–2 varieties of quinoa in the previous studies that exempted any scope of understanding existing cytological variations in the species. The latter reason stated here might also be true for the limited variations observed in the position of primary constrictions in the past studies. Chromosomes with only nearly median constrictions were observed by Palomino *et al.* (2008) and Bhargava *et al.* (2006), with only a single variety revealing 2 chromosomes with sub-median constrictions being reported by Bhargava *et al.* (2006). This was in contrast with the presence of sub–terminal constrictions in about 75% of the varieties of quinoa investigated in the present study revealing higher degree of intra–chromosomal variations (Table 1, Figure 3G). The number of chromosomes possessing secondary constrictions in each complement of all the cultivars in the present study, corroborated observations of Bhargava et al (2006), though contrasting observations of 2 pairs of secondary constrictions were reported by Palomino et al (2008). However, some other studies have reported absence of chromosomes with secondary constrictions (Cárdenas and Hawkes 1948; Gandarillas and Luizaga 1967; Giusti 1970; Gandarillas 1979). Chromosomes possessing secondary constriction had median or nearly median primary constriction and secondary constrictions in sub–median position. Gross chromosome morphology obtained in the present study, although showed significant variations in both chromosome length and constrictions, did not reveal any characteristic pattern that can be related to the geography or seed morphology of the studied cultivars.

Study of chromosomal characteristics forms the basis of cytotaxonomy that can be used to understand similarity or divergence among related species, and is based

on asymmetry indices calculated with the help of chromosome measurements (Levitzky 1931; Stebbins 1971). Since asymmetry is an expression of chromosomal morphology, it is important to compare karyotypes on appropriate statistical grounds and choose correct statistical parameters (Peruzzi and Altinordu 2014). However, there is a lack of general consensus on the specific karyotype asymmetry parameter to be utilized which necessitates use of multiple available parameters to conclude about the asymmetry status of a taxa and its comparison with relatives. Inter-chromosomal asymmetry increases with increasing difference between the lengths of smallest and largest chromosomes of a complement, and, are assessed by calculating A2 and CV_{CL}. Intra-chromosomal asymmetry increases with shift in centromeric position from median to terminal constriction in a complement. This is assessed by calculating the values of TF%, Syi, AsK%, A1, M_{CA} and CV_{CI} (Paszko 2006; Peruzzi and Eroğlu 2013).

In the present investigation, the different cultivars of quinoa showed significantly higher asymmetry than former studies as was evident by the karyotype asymmetry indices (Table 1). Stebbins asymmetry class, where all the cultivars of the present study were classified as 2B karyotype, endorsed more asymmetric karyotype than 1A and 1B obtained from 7 cultivars by Bhargava *et al.* (2006). AI, with an average value of 3.21±0.62 also indicate the same. However, calculation of these 2 indices are based on a combination of inter- and intra-chromosomal asymmetry, which has been suggested against in recent studies (Peruzzi and Eroglu 2013). TF% that decreased with increasing asymmetry ranged from 39.69–43.23% with an average of 41.29±0.86%, lower than previously reported values of 43.8–47.4% (Bhargava *et al.* 2006; Palomino *et al.* 2008) affirming higher asymmetry revealed in the present study. Similarly, evaluation of AR that ranged from 0.34 to 5.76 in the present study, but showed much less variation (1.00–1.86) revealing lesser asymmetry in the previous studies also imply presence of higher variation among the cultivars assessed in the present study (Bhargava *et al.* 2006; Palomino *et al.* 2008). Other asymmetry indices examined in the present study also supported the above contention as was evident by the calculated Pearson correlation. Inter–chromosomal and intra–chromosomal asymmetry indices revealed strong correlation within themselves (Table 2). TF% was found to be negatively correlated with other intra–chromosomal indices of A1 (r= −0.978), CV_{CI} (r= −0.770), M_{CA} (r= −0.991), Syi (r= −0.995) and Ask% (r= −1.000) justifying increasing intra-chromosomal asymmetry with decreasing value of TF%. A2 was positively correlated with CV_{CL} (r= 0.992) indicating increasing value with increasing differences in chromosome size. However, inter– chromosomal asymmetry indices were weakly correlated with intra–chromosomal indices, justifying the suggestion of not including both type of indices in a single analysis (Peruzzi and Eroglu 2013).

Both inter- and intra-chromosomal asymmetry are necessary to reveal correlation among related taxa. This is best represented by using bi-dimensional scatter plots that include one inter-chromosomal and one intra-chromosomal parameter in each axis of the plot (Peruzzi and Eroğlu 2013). Restricted distribution of cultivars in the scatter plots generated in the present study comparing the values of A1 versus A2, CV_{CI} versus CV_{CL}, and, M_{CA} versus CV_{CL} indicated high similarity among the cultivars with respect to chromosomal asymmetry (Figure 4). The plot indicated that, although the study revealed consistent differences within the karyotype of each cultivar, the characteristic distinctions were inadequate for cultivar identification, and not related to their geographical origin and seed morphology.

Overall, high inter- and intra-chromosomal variability in the quinoa cultivars examined here conclusively exhibited that much variation exists in this species that is yet to be explored exhaustively. During early domestication events, conspicuous divergence of domesticated

Table 2. Values of Pearson correlation analysis for intra- and inter-chromosomal asymmetry indices

	A1	A2	CV_{CL}	CV_{CI}	M_{CA}	TF	Syi	AsK
A1	1							
A2	−0.071	1						
CV_{CL}	−0.073	0.992*	1					
CV_{CI}	0.667*	0.110	0.118	1				
M_{CA}	0.985*	−0.067	−0.070	0.765*	1			
TF	−0.978*	0.070	0.079	−0.770*	−0.991*	1		
Syi	0.981*	−0.121	−0.125	0.745*	0.990*	−0.995*	1	
AsK	0.977*	−0.070	−0.079	0.770*	0.991*	−1.000*	0.994*	1

*Correlation is significant at 0.01 level.

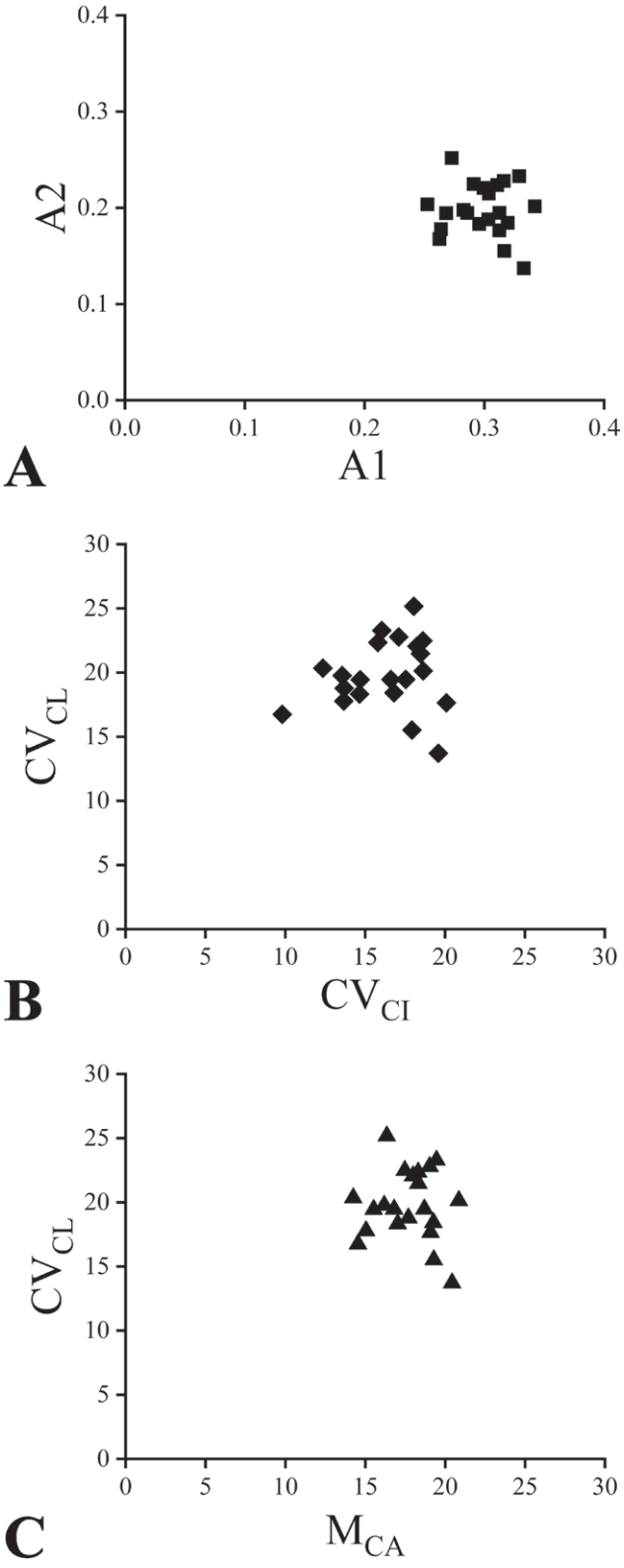

Figure 4. Scatter diagrams for the *Chenopodium quinoa* cultivars of (A) A1 against A2 (B) CV_{CI} against CV_{CL} (C) M_{CA} against CV_{CL}.

species from their wild relatives resulted from artificial selection and controlled reproduction, especially cross–fertilization, by human intervention, that ended in genetic bottleneck, thereby reducing genetic diversity of cultivated plants with respect to their wild counterparts (Tanksley and McCouch 1997; Cornille *et al.* 2014). Along with this, early farmers selected few individuals with more desirable traits for next generation cultivation with little effort of conserving the unselected materials that were being pushed to oblivion, thereby causing severe loss of genetic diversity and limiting the gene pool of present day domesticated plants (Pan *et al.* 2016). In recent years, cultivation of local varieties of quinoa has been largely neglected because of increased pressure on local farmers for production of certified high–yielding quinoa varieties in international quinoa market (Salazar *et al.* 2019). The inter–varietal similarity and stability of chromosomal characteristics observed in our studied quinoa cultivars might be an indication of the bottleneck experienced by the plants due to their long domestication history of over 7,000 years (Jarvis *et al.* 2017; Salazar *et al.* 2019), that had allowed enough time to impart homogeneity and stability in their chromosomal structures. Loss of diversity in local quinoa gene pool in this way will pose serious threat in future breeding programs for quality improvement that necessitates an imperative exploration of chromosomal variability (Jarvis *et al.* 2017; Salazar *et al.* 2019). This entails an imperative investigation of inter–varietal chromosomal diversity in quinoa that would include chromosomal fluorescent staining techniques, like FISH, incorporating a large number of cultivars encompassing varieties growing on the distinct eco–geographical regions of quinoa cultivation, primarily from the highland and the coastal lowland ecotypes. It will provide a comprehensive basis for evaluation, selection, conservation and maintenance of existing germplasm, thereby assisting in future breeding programs.

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Cytogenetic and molecular studies of the Egyptian *Capsella bursa-pastoris* (Brassicaceae)

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Abstract. *Capsella bursa-pastoris* (Brassicaceae) is one of the most successful tetraploid species in the world. It showed high morphological diversity within Egyptian populations. Morphological investigations of herbarium specimens and fresh collected populations grouped them under three distinguished morphotypes (Lobed "L"; Simple "S" and Lobed-Simple "LS") depending mainly on the basal leaves structure. This high degree of phenotypic variation has received our critical attention. Until recently, the previous studies on *C. bursa-pastoris* attributed its phenotypic variation to environmental factors. But in Egypt, these three morphotypes were traced in mixed populations along with the species geographical range, so the environmental factors have no influence on their distribution or phenotypic variation. Accordingly, our primary concern in this study was to determine the factors controlling this variation. The cytogenetic studies revealed that the three identified morphotypes are three distinct genotypes with three different chromosome numbers: 2n=2x=16 (diploid) for "L"; 2n=3x=24 (triploid) for "S"; 2n=4x=32 (tetraploid) for "LS". The triploid genotype "S" showed rare occurrence among the studied populations and is postulated to be a new record of a hybrid in Egypt. Karyotyping of the three genotypes showed significant differences in the genome and chromosomes relative lengths. Molecular study using cpSSR technique supported the cytogenetic results and differentiated the three studied genotypes. The retrieved results revealed that the phenotypic diversity within the Egyptian *C. bursa-pastoris* populations is genetically controlled.

Keywords: *Capsella bursa-pastoris*, cytogenetic studies, genotypes, karyotyping, molecular study, phenoplasticity.

INTRODUCTION

Brassicaceae (Cruciferae) or mustard family is one of the largest Angiosperm families, it comprises 3977 species and 341 genera and 52 tribes (Kiefer *et al.*, 2014). One of the most important genera in the Brassicaceae is genus *Capsella* Medik. Molecular systematic studies confirmed that genus *Capsella* belongs to the tribe Camelineae (Neuffer *et al.*, 2014). This genus is an excellent model for molecular evolutionary studies due to its phylogenetic relations within the Brassicaceae. Studying its genetics, speciation and sympatric distribution is important for agricultural matters.

Genus *Capsella* is represented worldwide by five species: the two self-compatible tetraploid *C. bursa-pastoris* (L.) Medik and *C. thracica* Velen

(2n=4x=32); the self-incompatible diploid *C. grandiflora* (Fauche & Chaub) Boiss; the two self-compatible diploid *C. rubella* Rent. and *C. orientalis* Klokov (Hurka *et al.*, 2012; Neuffer *et al.*, 2014). These species differ greatly in their geographical distribution, where, *C. grandiflora* is limited to northwestern Greece and Albania; *C. rubella* has a broad Mediterranean / central European distribution; *C. orientalis* is found from Eastern Europe to Central Asia (Hurka *et al.*, 2012); *C. thracica* is endemic to Bulgaria (Neuffer *et al.*, 2014).

Capsella bursa-pastoris (Shepherd's Purse) is an annual to biennial species, extremely variable in size and leaf form, distinguished by terminal and axillary raceme inflorescences. Its silicula fruit is obcordate-obtriangular in shape (Amer *et al.*, 2019). This species is the second most common flowering plant in the world (Zhou *et al.*, 2001), grows as a common weed of agriculture in almost all countries of the world from tropical to subarctic habitats (Holm *et al.*, 1979), and shows a high phenotypic plasticity (Korsmo, 1954; Holzner and Numata, 1982). Accordingly, the evolution of polyploidy and weediness in *C. bursa pastoris* is interesting to agricultural research (St Onge, 2010).

Many taxonomic studies were carried out on this species depending on morphological characters and resulted into many species, subspecies, varieties, microspecies, biotypes, and segregates (Aksoy *et al.*, 1998; Aksoy *et al.*, 1999; Neuffer, 2011).

A considerable amount of literature has attributed the phenotypic variation in *C. bursa-pastoris* to environmental or geographical factors like seasonality, temperature, shade, rainfall, latitudinal and altitudinal gradients (Almquist, 1929; Neuffer, 1989; Neuffer and Bartelheim, 1989; Stace, 1989; Neuffer, 1990; Aksoy, 1996; Aksoy *et al.*, 1999; Neuffer and Hoffrogge 2000).

In Egypt, *Capsella* is a monospecific genus represented by *C. bursa-pastoris* (Boulos, 1999). The field study and morphological investigations of this species – based on the leaves, inflorescence and fruit characters – showed the presence of high degree of phenotypic variation and revealed the presence of three morphotypes namely: Lobed "L", Simple "S" and Lobed-Simple "LS" (Amer *et al.*, 2019). These three morphotypes were traced in mixed populations along with the species geographical range, so the environmental factors have no influence on their distribution or phenotypic variation.

Therefore, this study aims to determine the factors controlling the phenotypic variation of *C. bursa-pastoris* morphotypes through cytogenetic and molecular studies to clarify the impact of genetic diversity on their phenoplasticity.

MATERIALS AND METHODS

Morphological study

The morphological investigations of *C. bursa-pastoris* were carried on 36 old populations deposited as herbarium specimens in Cairo University Herbarium (CAI) and Assiut University Herbarium (ASTU). In addition to 66 fresh populations collected from Menoufia (Abu Sleem village), Faiyum (Sinnuris district, El Siliene) and El Saff regions during our field work conducted in 2016-2018. The studied specimens (old & fresh) from different distribution localities are shown in Table 1. From 66 fresh populations, 25 specimens/ population were undergone morphological investigations using different morphological criteria of leaves, inflorescence and fruit. Acronyms of herbaria follow Thiers (2019).

The morphological investigations distinguished three morphotypes of *C. bursa-pastoris* in all the studied populations namely: (L) Lobed, (S) Simple and (LS) Lobed-Simple (Amer *et al.*, 2019).

Cytogenetic studies

Sample preparation

For cytogenetic studies, the specimens collected from Faiyum region (marked with * in Table 1) were selected to nullify the environmental factors. Seeds of 30 specimens representing the three morphotypes (10/ morphotype) were collected, soaked in distilled water for 2 hours, and germinated at room temperature. Root tips of about 1 cm length were treated with colchicine ($C_{22}H_{25}NO_6$, 0.025 %) for 2 hours at room temperature, and washed thoroughly with distilled water. Fixation was done using ethyl alcohol: glacial acetic acid (3:1, v/v). Samples were washed thoroughly with water and hydrolyzed using 1 N HCl at 64° C for 5 minutes. The slides were prepared by squashing the root tips using 45% acetic acid and stained with aceto orcein solution.

Chromosome count and karyotyping

Chromosome count was performed on mitotic metaphase cells. For each morphotype, ten clearly observable metaphase cells from ten individuals were selected and photographed using standard and high resolution automated karyotyping software processing (Leica CW4000). Metaphase chromosomes of each morphotype were placed in pairs, arranged and numbered in order of size, with keeping in view the centromere position to consti-

tute a karyotype. The length of the short arm (p) and the long arm (q) was measured for each chromosome, and the total length (TL=p+q) was calculated. The relative length (RL) of the chromosomes (TL / sum TL × 100) and the mean relative length (MRL) of each chromosome pair were calculated. The centromeric index (CI) was estimated by (P / TL x 100), the mean centromeric index (MCI) was calculated to represent the centromeric index value of a particular chromosome pair, then the chromosomes were classified according to Levan *et al.* (1964).

Molecular study

From the same 30 specimens collected from Faiyum region (Table 1) for cytogenetic studies, 14 specimens representing the three morphotypes were chosen for molecular study (4 Lobed, 4 Simple and 6 Lobed-Simple).

DNA extraction

A total genomic DNA was extracted from 1 g young leaves using CTAB (cetyl-trimethyl ammonium bromide) extraction buffer procedure described by Doyle and Doyle (1990) and modified by Allen *et al.* (2006).

PCR reactions and data analysis

For each 25 µl PCR reaction, add 12.5 µl Dream Taq Green PCR Master Mix (2X), 1 µl Forward primer (5′-GCC TAC CGC ATC GAA ATA GA-3′), 1 µl Reverse

Table 1. Collected specimens of *Capsella bursa-pastoris* (L.) Medik. in Egypt with their geographical distribution (arranged from North to South).

Collection & Herbarium	Date	Longitude	Latitude	Locality
Amer 8312 (CAI)	18.1.1987	30°26'33"	31°25'15"	Beheira Province, Rosetta
Fahmy 963 (CAI)	2.5.1988	27°14'55"	31°21'23"	Mersa Matruh, El Sallum road
Fayed & El Naggar s.n. (ASTU)	15.3.1984	30°00'44"	31°17'00"	Alexandria, El Montazha
Amer 16225 (CAI)	6.3.1988	30°32'58"	31°12'17"	Beheira Province, Mahmudiya
Abdel Fattah & Abdel Aziz s.n. (CAI).	19.3.1974	31°23'00"	31°01'53"	El Mansoura
Gun Romée 443 (CAI)	12.3.1968	30°55'33"	30°47'13"	Tanta
Amer 1515 (CAI)	18.3.1982	31°48'59"	30°43'19"	Sharkiya, Faqus
G. Täckholm s.n. (CAI)	7.1.1927	31°12'46"	30°41'40"	Barrage (Zifta)
El Naggar s.n. (ASTU)	30.1.1985	31°11'11"	30°27'19"	Banha, Kafor Mousa
El Bakry 2708 (CAI)	29.4.1981	31°33'43"	30°24'57"	Bilbeis
Chrtek, Kosinova & Slavikova s.n. (CAI)	4.4.1977	31°17'00"	30°12'01"	Bahtim
El Batanony s.n. (CAI)	7.2.1957	31°14'14"	30°07'26"	El Menoufia
Amer *et al.* s.n. (CAI)	27.1.2017	31°12'54"	30°06'45"	El Menoufia, Abu Sleem village
El Hadidy s.n. (CAI)	17.1.1952	31°11'58"	30°04'52"	Imbaba
El Hadidy s.n. (CAI)	12.1.1956	31°12'27"	30°01'39"	Giza, Faculty of Science farm
Taher El sayed s.n. (CAI)	19.11.1926	31°11'45"	30°01'12"	Giza, in clover fields
Chrtek & Kosinova s.n. (CAI)	13.4.1971	31°12'29"	30°01'05"	Giza, Faculty of Agriculture farm
Chrtek & Kosinova s.n. (CAI)	1.4.1971	31°13'12"	30°00'35"	Giza, El Harraniya village
Chrtek, Kosinova & Imam s.n. (CAI)	27.4.1967	31°15'48"	29°35'21"	El Saff, fields along the road
Amer *et al.* s.n. (CAI)	23.4.2018	31°15'17"	29°34'57"	El Saff
Abd El Ghani 5820 (CAI)	13.3.1983	30°51'27"	29°24'48"	Faiyum, Sinnuris district, El Siliene
Amer *et al.* s.n. (CAI)	27.1.2017	30°51'27"	29°24'48"	*Faiyum, Sinnuris district, El Siliene
Abd El Ghani 5234 (CAI)	8.3.1983	30°48'56"	29°21'20"	Faiyum district, Beni Saleh
Abd El Ghani 5320 (CAI)	8.3.1983	30°27'11"	29°19'16"	Faiyum district, in clover fields
Fayed *et al.* s.n. (ASTU)	2.5.2010	34°18'25"	27°56'48"	Southern Sinai, Farsh Elias
Zareh & Fayeds.n. (ASTU)	5.12.1990	31°12'05"	27°10'20"	Assiut, Sohag East road
Zareh s.n. (ASTU)	5.12.1990	31°20'18"	27°02'44"	Assiut, El- Matmar
Zareh & Fayed s.n. (ASTU)	30.1.1991	31°22'02"	26°57'05"	Assiut, Sedfa
Zareh s.n. (ASTU)	28.2.1962	32°00'10"	26°14'08"	El-Balliana, Sohag

*Specimens subjected to cytogenetic and molecular studies.

primer (5′- CAA GAA AGT CGG CCA GAA TC-3′), 2 µl Template DNA, and complete to 25 µl by water (nuclease-free).

The PCR was performed using the recommended thermal cycling conditions: one cycle of initial denaturation at 95°C for 5 minutes, 35 cycles of denaturation at 95°C for 45 seconds followed by annealing at 57°C for 45 seconds then extension at 72°C for 60 seconds, and one cycle of final extension at 72°C for 10 minutes.

The reaction products were separated by electrophoresis on 1.6 % agarose gel in 1x TBE buffer and run in the same buffer at 100 V for 1 hour, and visualized by staining with 0.5 µg/ml of ethidium bromide and photographed under UV light.

The cpSSR locus ATCP31017 was sequenced and DNA was amplified using the previously mentioned primers (Castro *et al.*, 2014). The amplified fragments were sequenced in ABI377 DNA sequencer (ABI, USA). Then BLAST programs were used for searching DNA databases for sequence similarities. Mega software was used to carry out multiple sequence alignment and calculate genetic distances among studied taxa. Neighbour-joining dendrogram was constructed showing the genetic relationships among 14 specimens of the three studied genotypes.

RESULTS

Morphological diversity

The morphological investigations and taxonomic revision of 36 old herbarium specimens and 66 recently collected populations of *C. bursa-pastoris* based on 25 morphological characters including plant height, basal and cauline leaves features, as well as inflorescence and fruit characters (Amer *et al.*, 2019). The most differential characters were that of the basal leaves. The results of that study (Amer *et al.*, 2019) revealed the presence of three morphotypes in Egypt namely: Lobed "L" with all basal leaves are lobed, Simple "S" in which all basal leaves are simple, and Lobed-Simple "LS" in which basal leaves are mixed lobed with simple (Figure 1).

The three identified morphotypes were co-distributed and traced in the field as mixed populations along with the species geographical range. Where, the "LS" morphotype was the most common type and showed the highest phenotypic variation, while the "S" morphotype showed rare occurrence in all studied localities. The environmental factors such as shading, temperature, soil type and rainfall showed no influence on the distribution of these morphotypes (Amer *et al.*, 2019).

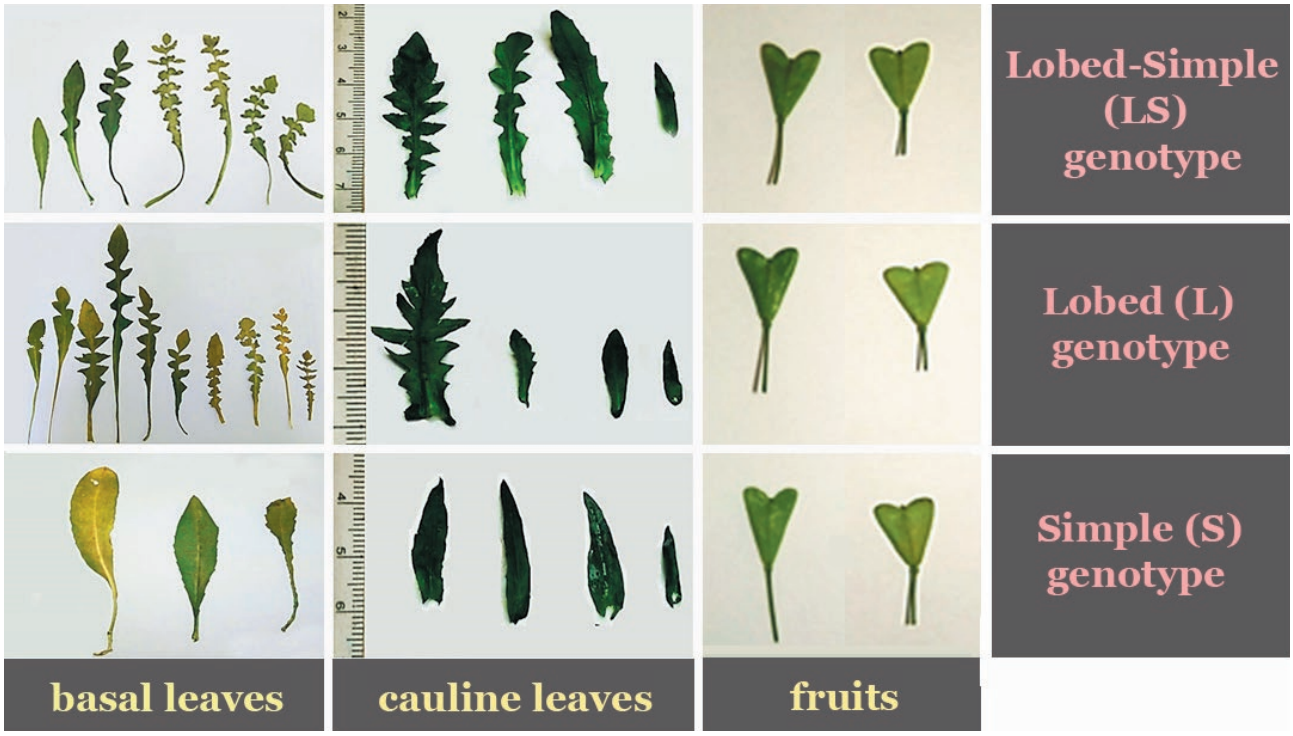

Figure 1. Morphological diversity within the Egyptian *C. bursa pastoris* genotypes; L: Lobed, **S:** Simple, **LS:** Lobed-Simple.

Cytogenetic analysis

Chromosome number

Chromosome count for each morphotype of *C. bursa-pastoris* was done in the mitotic metaphase (Figure 2). The three studied morphotypes recorded three different chromosome numbers. Accordingly, they are treated as three distinct genotypes. The Lobed "L" genotype has diploid chromosome set of 2n=2x=16. The Simple "S" genotype has triploid chromosome set of 2n=3x=24 with minor aneuploidy in chromosome no. 6 and 8 (2n=3x-2=22). This triploid genotype is recorded for the first time in Egypt. While the Lobed-Simple "LS" genotype recorded the presence of tetraploid chromosome set of 2n=4x=32 (Figure 2).

Karyotype analysis

The karyotyping data of these three genotypes are provided in Figure 3 and Table 2. The retrieved results showed that the chromosomes are small in size, the total genomic length ranges from 43.48 µm in the Lobed "L" genotype to 67.76 µm in Lobed-Simple "LS" genotype, while the Simple "S" genotype has an intermediate value of 61.46 µm.

Furthermore, the chromosomes are highly variable referring to their mean relative length (MRL), as shown in Figure 4. The chromosome pair 1 is the longest in the three genotypes, its length ranges from 3.21 µm in Lobed-Simple "LS" genotype to 4.48 µm in Simple "S" one, and its mean relative length (MRL) ranges from 4.74% in Lobed-Simple "LS" genotype to 7.99% in Lobed "L" one. The length of the shortest chromosome pair 8 ranges from 1.54 µm in Simple "S" to 1.74 µm in Lobed "L, and its mean relative length (MRL) ranges from 2.35% in Simple "S" genotype to 3.99% in Lobed "L" with an intermediate value of 2.52% in Lobed-Simple "LS" genotype.

The eight chromosome pairs were grouped based on the centromere position into four types: acrocentric, metacentric, submetacentric and subtelocentric (Table 2). The chromosome pairs from 1 to 4 are metacentric in all the studied genotypes. The chromosome pair no. 5 is metacentric in genotypes "L" and "S", while it is acrocentric in "LS" genotype. The chromosome pair 6 is acrocentric in "L" genotype, while it is submetacentric in "S" and "LS" genotypes. The chromosome pair 7 appeared acrocentric in "L" genotype, submetacentric in "S" genotype, and metacentric in "LS" genotype. The chromosome pair 8 is metacentric in "L" and "S" genotypes, while subtelocentric in "LS" genotype.

Molecular analysis

Gel electrophoresis of the PCR amplification products of 14 studied specimens (4 Lobed, 4 Simple and 6 Lobed-Simple) produced 14 bands of good quality.

The statistical results of cpSSR locus ATCP31017 sequences developed a Neighbour-joining dendrogram (Figure 5) that separated the genotypes "L", "S", and "LS" into three genetic clusters. The first cluster included four specimens (L1-4) that represented the Lobed "L" genotype (2n=16). In this cluster, specimens L1 and L3 showed high genetic similarity to each other. The second cluster included also four specimens (S5-8) that represented the Simple "S" genotype (2n=24). In this cluster, specimens S5 and S7, and specimens S6 and S8 showed high genetic similarity to each other. The third cluster included six specimens (LS9-14) that represented the Lobed-Simple "LS" genotypes (2n=32). In this cluster, specimen LS9 showed the lowest genetic similarity

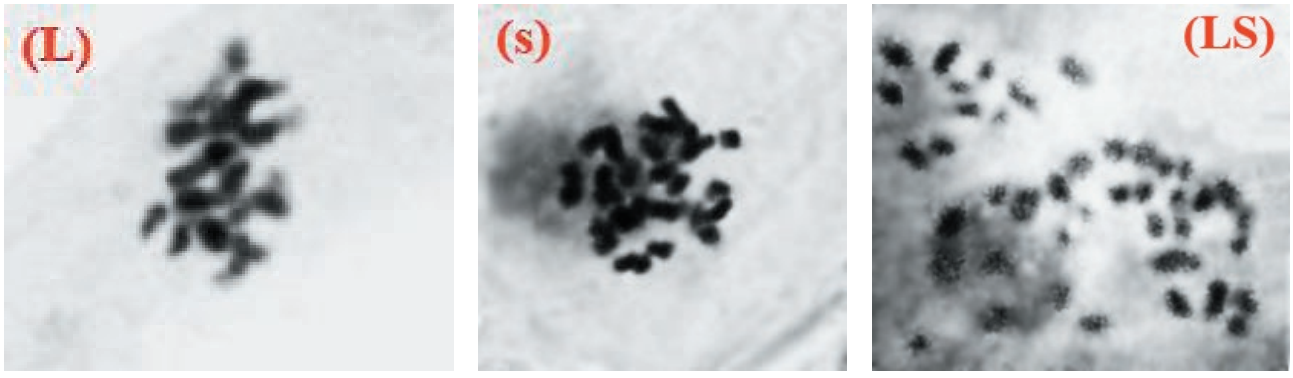

Figure 2. Photomicrographs of well spread mitotic metaphase in the three genotypes of *C. bursa pastoris*: **L:** Lobed diploid with 2n=2x=16; **S:** Simple triploid with 2n=3x=24; **LS**: Lobed-Simple tetraploid with 2n=4x=32.

Table 2. Karyotyping data for the studied *C. bursa pastoris* genotytpes, **L:** lobed, **S:** simple, **LS:**lobed-simple, **A:** Acrocentric, **M:** Metacentric, **SM:** Submetacentric, **ST:** Subtelocentric.

	L	S	LS
Long arm (q) µm			
1	1.74±0.05	2.74±0.63	1.63±0.05
2	1.58±0.00	1.97±0.12	1.29±0.16
3	1.58±0.10	1.37±0.10	1.10±0.26
4	1.37±0.10	1.23±0.04	1.05±0.10
5	1.37±0.00	1.19±0.09	1.63±.38
6	2.32±0.00	1.51±0.25	1.34±0.24
7	2.21±0.00	1.23±0.23	0.95±0.15
8	1.08±0.50	0.84±0.49	1.35±0.22
Short arm (p) µm			
1	1.74±0.05	2.10±0.32	1.58±0.11
2	1.58±0.00	1.86±0.16	1.21±0.16
3	1.53±0.05	1.33±0.11	0.95±0.11
4	1.32±0.05	1.19±0.04	1.00±0.11
5	1.21±0.05	1.16±0.10	0.22±0.10
6	0.26±0.15	0.70±0.39	0.45±0.44
7	0.21±0.00	0.74±0.35	0.84±0.06
8	0.66±0.34	0.70±0.39	0.36±0.15
Total length (p+q) µm			
1	3.48±0.10	4.84±0.95	3.21±0.16
2	3.16±0.00	3.83±0.28	2.5±0.32
3	3.11±0.15	2.70±0.20	2.05±0.37
4	2.69±0.15	2.42±0.08	2.05±0.21
5	2.58±0.05	2.35±0.19	1.84±0.48
6	2.58±0.15	2.21±0.64	1.79±0.58
7	2.42±0.00	1.97±0.55	1.79±0.21
8	1.74±0.54	1.54±0.79	1.71±0.37
Mean relative length (MRL)			
1	7.99	7.38	4.74
2	7.27	5.83	3.69
3	7.14	4.12	3.03
4	6.18	3.69	3.03
5	5.94	3.59	2.72
6	5.94	3.37	2.64
7	5.57	2.99	2.64
8	3.99	2.35	2.52
Mean centromeric index (MCI)			
1	50	44.41	49.13
2	50	48.55	48.41
3	49.23	49.18	45.67
4	49.02	49.17	48.82
5	46.88	49.25	12.3
6	9.94	30.69	25.01
7	8.68	36.63	47.16
8	40.11	47.53	21.93
Type			
1	M	M	M
2	M	M	M
3	M	M	M
4	M	M	M
5	M	M	A
6	A	SM	SM
7	A	SM	M
8	M	M	ST

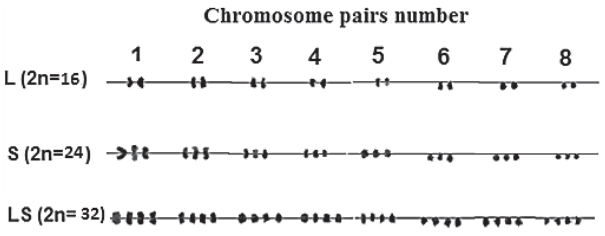

Fig. 3. Karyotypes of *C. bursa-pastoris* genotytpes. L: Lobed diploid (2n=2x=16); **S:** Simple triploid (2n=3x=24); **LS:** Lobed-Simple tetraploid (2n=4x=32).

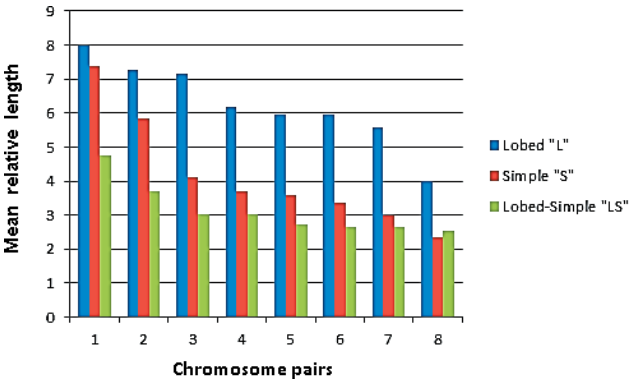

Fig. 4. Mean relative length (MRL) of each chromosome pair in the three studied genotypes of *C. bursa-pastoris.*

with other specimens. While specimens LS10 and LS12, also LS13 and LS14 showed high genetic similarity to each other.

The DNA sequences of the studied 14 specimens of *C. bursa-pastoris* were registered on the National Center for Biotechnology Information (NCBI) under the following accession numbers MN602606, MN602607, MN602608, MN602609, MN602610, MN602611, MN602612, MN602613, MN614131, MN614132, MN614133, MN614134, MN614135 and MN614136.

	L1	L2	L3	L4	S5	S6	S7	S8	LS9	LS19	LS11	LS12	LS13	LS14
L1														
L2	0.0258													
L3	0.0205	0.0361												
L4	0.0255	0.0415	0.0279											
S5	0.0361	0.0685	0.0457	0.0602										
S6	0.0306	0.0515	0.0415	0.0531	0.0395									
S7	0.0363	0.0685	0.0462	0.0609	0.0315	0.0536								
S8	0.0306	0.0495	0.0402	0.0531	0.0395	0.0351	0.0541							
LS9	0.1682	0.1636	0.1651	0.1835	0.1553	0.1591	0.1532	0.1622						
LS10	0.0573	0.0856	0.0797	0.0797	0.0721	0.0756	0.0735	0.0921	0.1429					
LS11	0.0567	0.0822	0.0736	0.0689	0.0602	0.0715	0.0621	0.0852	0.1441	0.0622				
LS12	0.0575	0.0861	0.0807	0.0802	0.0725	0.0763	0.0742	0.0932	0.1296	0.0563	0.0672			
LS13	0.0518	0.0755	0.0696	0.0672	0.0567	0.0631	0.0546	0.0818	0.1468	0.0714	0.0702	0.0815		
LS14	0.0515	0.076	0.0685	0.0664	0.0565	0.0625	0.0541	0.0812	0.1521	0.0718	0.0711	0.0822	0.0363	

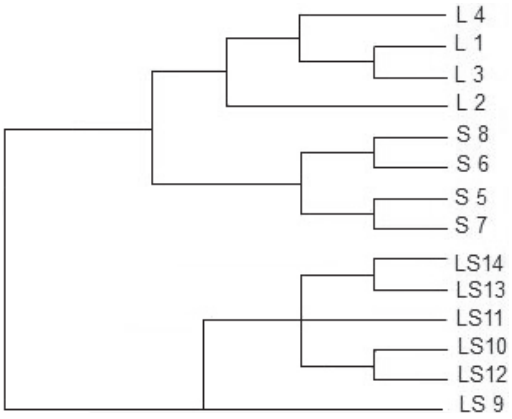

Fig. 5. Diagonal matrix of the genetic distances and Neighbour-joining dendogram showing the genetic relationship among 14 specimens of the three studied genotypes (L, S and LS).

DISCUSSION

The taxonomic revision of *Capsella bursa-pastoris* in Egypt revealed the presence of a high degree of phenoplasticity in all the studied populations (old and fresh) and resulted in three distinctive morphotypes ("L" Lobed, "S" Simple and "LS" Lobed-Simple) based mainly on the basal leaves structure (Amer *et al.*, 2019). These morphotypes were traced as mixed populations in all the studied localities (Table 1), so the environmental factors have no influence on their distribution or phenoplasticity (Amer *et al.*, 2019). However, positive correlation between the degree of genetic heterogeneity and environmental variability was reported by Aksoy *et al.* (1998), Neuffer *et al.* (1999), Neuffer and Hurka (1999) and Castro *et al.* (2014). In addition to Han *et al.* (2015) who reported that *C. bursa-pastoris* populations from different regions are polymorphologically different.

The study of genetic diversity of *C. bursa-pastoris* in Egypt has received little attention so far. In this study, we tried to elucidate the role of genetic diversity within the Egyptian morphotypes in their phenoplasticity.

The chromosome counting of the identified morphotypes recorded three different chromosome numbers (2n=16 for Lobed "L", 3n=24 for Simple "S", 4n=32 for Lobed-Simple "LS"), so they are treated here as genotypes. Shull (1909) was the first to distinguish between four biotypes with different leaf types (simplex, rhomboidea, tenuis, and heteris) and cleared the correlation between leaf shape and chromosome number. He recorded the tetraploid number for simplex and rhomboidea biotypes, while heteris and tenuis were diploid. Nonetheless, the Egyptian morphotypes are not equivalent to Shull's biotypes (Amer *et al.*, 2019).

Worldwide, the tetraploid *C. bursa-pastoris* (2n=4n=32) is one of the most successful plants that have high polymorphic level (Han *et al.*, 2015). Similarly in Egypt, the tetraploid "LS" genotype is the most common and diverse type with a wide range of leaf forms on the same plant (Amer *et al.*, 2019). This is supported by Shull (1929), Löve and Löve (1956), Davis (1965), Raj (1965), Hsu (1968), Svensson (1983) and Hurka (1984).

The diploid chromosome number (2n=2x=16) recorded in Lobed "L" genotype, was early recorded in Europe by Bosbach and Hurka (1981), in Greece by Svensson (1983), and in Kashmir by Jeelani *et al.* (2013).

Although *C. bursa-pastoris* is a self-compatible species, different percentages of outcrossing were recorded by many authors: Aksoy *et al.* (1998) recorded 1-2%, Hurka *et al.* (1989) recorded 3-12%, and Hurka and Neuffer (1997) recorded up to 20%.

In reviewing the literature, no data were found on the triploid "S" genotype (2n=3x=24) which is recorded for the first time in Egypt. Its rare presence within *C. bursa-pastoris* populations comparing with the other two genotypes (Amer *et al.*, 2019) may support its hybrid origin. The lack of chromosome no. 6 and 8 (aneuploidy, 2n=3x-2=22) in some individuals of this genotype supports our postulation. Bretagnolle and Thompson (1995) cleared that the triploid offspring are typically sterile due to problems in chromosomal pairing and segregation during meiosis, which may cause aneuploid gametes and result in sterility.

Karyotype analysis showed that the chromosomes are small in size, this result agrees with Schmidt and Bancroft (2011) who reported that mitotic chromosomes of crucifer species are generally very small in size. The karyotyping of the three genotypes (Fig. 3 & Table 2) showed distinctive variations within genotypes in the

genome and chromosomes mean relative lengths (MRL) in addition to the centromere position. Our results are supported by Guerra (2008) who claimed that the karyological data in taxonomy contribute to evaluate the genetic relationships among species or populations and lead to better understanding of the way they diverged from each other.

As reported by Amer *et al.* (2019), the "LS" populations were large in size, up to 80 cm length with inflorescence up to 75 cm, while "L" and "S" populations were small in size (up to 50 cm long with inflorescence up to 40 cm). These results indicate that the growth rate of tetraploid populations is greater than that of other genotypes, and agree with Neuffer's finding (1989) that the rate of growth was greater for the tetraploid groups. On the other hand, the small size of the diploid "L" and the triploid "S" genotypes can be explained by deletion of redundant genes which can result in downsizing of the genome, as reporteded early by Devos *et al.* (2002), Blanc and Wolfe (2004), Vitte and Bennetzen (2006).

The molecular results achieved by sequencing the cpSSR locus ATCP31017 of 14 specimens of *C. bursa-pastoris* genotypes support the cytogenetic results, where the neighbour-joining dendrogram (shown in Figure 5) separated the studied genotypes "L", "S", and "LS" into three distinctive genetic clusters. The first cluster included four specimens for the diploid "L" genotype. In this cluster, specimens L1 and L3 showed high genetic similarity with each other, this result may be reflected from high morphological similarity (both specimens had plant length up to 50 cm and inflorescence up to 40 cm and similar cauline leaves). The second cluster included four specimens for the triploid "S" genotype. In this cluster, specimens S5 and S7 showed high genetic similarity reflected from morphological similarities with each other (plant length up to 50 cm, its inflorescence up to 40 cm). Moreover, specimens S6 and S8 showed high genetic similarity as both samples had very small plant size (stem length was up to 20 cm, its inflorescence was up to 15 cm). The third cluster included six specimens for the tetraploid "LS" genotype. In this cluster, specimen LS9 had the lowest genetic similarity with the other specimens. This may be due to its very large size (stem length was up to 80 cm, its inflorescence was up to 75 cm) and presence of both simple and lobed cauline leaves on the same plant. On the other hand, specimens LS10 and LS12 were genetically similar (both specimens had large plant size and simple cauline leaves). Also, specimens LS13 and LS14 were genetically similar, as both specimens had small plant size and simple cauline leaves. The Lobed-Simple "LS" genotype with the highest phenotypic plasticity (as shown in Fig. 1), also showed high genetic diversity (Fig. 5). The position of the triploid "S" genotype in the obtained dendrogram is supporting our postulation that it is a hybrid between "LS" and "L" genotypes. Together these results provide important insights about the correlation of the phenotypic diversity within the Egyptian *C. bursa-pastoris* and the genetic diversity.

CONCLUSION

The phenotypic diversity within the Egyptian *C. bursa-pastoris* populations is genetically controlled. The three studied morphotypes represent distinct genotypes. The environmental factors have no effect on their phenoplasticity. The postulated hybrid (triploid genotype) may suffer from sterility and disappear in the near future.

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Genome size and chromosome number of *Psidium friedrichsthalianum* (O. Berg) Nied ("Cas") in six populations of Costa Rica

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Abstract. *Psidium friedrichsthalianum* (O. Berg) Nied is a species found from southern Mexico, Central America; and there are reports that it is also found in Venezuela and Ecuador. It is a common fruit component of the Costa Rican diet, and it is valued industrially for its high content of polyphenols, mainly proanthocyanidins (PACs). This crop is not completely domesticated and there are no improved varieties produced through plant breeding. Genome size or ploidy levels have not been investigated in Costa Rican populations of *Psidium friedrichsthalianum*. Information about chromosome number and genome size is paramount for plant breeding strategies. Therefore, the main objective of our study was to determine chromosome number using pollen meiocytes and genome size by flow cytometry in six populations of *P. friedrichsthalianum* in Costa Rica. We found x = 11 bivalent chromosomes in all meiocytes analysed, classifying these populations as diploid. All populations had an average nuclear DNA content of 2C = 1.960 ± 0.005 pg. No statistically significant differences in nuclear DNA content were found among populations. We conclude that the consistency in chromosome number and genome size among populations suggests a common origin among them. Our estimates of the number of chromosomes and genome size of *P. friedrichsthalianum* determined in this study will be essential for future breeding programs, hybridization practices and development of QTL (Quantitative Trait Loci).

Keywords: ploidy, fluorescent microscopy, 2C nuclear DNA, flow cytometry, Costa Rican Guava, plant breeding.

INTRODUCTION

Psidium friedrichsthalianum (O. Berg) Nied is a tropical species in the family Myrtaceae, subfamily Myrtoideae, tribe Myrteae (Lucas *et al.* 2019); commonly known as "Cas", "Sour Guava" or "Costa Rican Guava". It is a medium-sized tree with reddish branches and abundant foliage of intense green color. Flowers are perfect, possibly allogamous and pollination is performed by bees and occasionally by hummingbirds (Barahona and Rivera 1995). Fruits are fleshy globose berries, between 5 and 10 cm in diameter with a greenish to yellow exocarp and a very distinct soft and acidic pulp. In addition, it is presumed that its center of origin is in Costa Rica (Barahona and Rivera 1995; Rojas-Rodríguez and Torres-Córdoba 2013). "Cas" fruits are characterised by abundant polyphenol content, mainly proanthocyanidins (PACs); these metabolites have important antioxidant, anti-inflammatory, antimicrobial and vasodilatory properties (Cuadrado-Silva *et al.* 2017; Flores *et al.* 2013; Rojas-Garbanzo *et al.* 2019; Granados-Chinchilla *et al.* 2016; González *et al.* 2012). Vasconcelos *et al.* (2019) described the chemical composition and allelopathic properties of essential oils extracted from *P. friedrichsthalianum*, suggesting that this oil may be used as a natural weed control comparable in efficacy, to synthetic herbicides. This fruit is considered an important resource due to its photochemical properties; however, few studies have been conducted on this tropical fruit.

The germplasm of *P. friedrichsthalianum* in Costa Rica has not yet been genetically characterized, however, Srivastava (1977), reported this species as diploid (2n = 2x = 11), while Hirano (1967) reported tetraploid and hexaploid individuals in Central America samples. The diversity in chromosomal number previously reported for *P. friedrichsthalianum* may be a consequence of its ongoing domestication process. Information on chromosome numbers in the Myrtaceae is generally scarce, the fairly small chromosomes found in this taxonomic group, which rarely exceed 2 mm (Costa 2004), may curb chromosome determination. Presently, genome sizes have been reported for *Psidium acutangulum, Psidium cattleianum, Psidium guajava* L. (white cultivar), *Psidium guajava* L. (red cultivar), *Psidium guineense* and *Psidium grandifolium* (Costa and Forni-Martins 2006b, Costa *et al.* 2008; Machado-Marques *et al.* 2016; Coser *et al.* 2012; Souza *et al.*2015) (Table 1). However, the genome size or the 2C value of *P. friedrichsthalianum* have not been analyzed yet.

Estimates of the number of chromosomes and genome size for *P. friedrichsthalianum* are essential for the design of effective improvement strategies, such as hybridization practices, the development of QTL (Quantitative Trait Loci), as well as to better understand the effects of inbreeding and heterosis (Birchler 2013; Wash-

Table 1. Chromosomes number and genome size from different species of *Psidium* and *Eucalyptus* (Myrtaceae) determined in previous studies. The content of holoploid nuclear DNA (2C, pg DNA) and the content of monoploid DNA (1C, pg DNA) are also provided.

Species	2n	Ploidy level	Nuclear DNA content			Reference
			2C (pg)	1C (pg)	1C (Mbp)*	
Genus *Eucalyptus*						
Eucalyptus microcorys	22	2x	1.040	0.520	508.56	Almeida-Carvalho *et al.*(2017)
Eucalyptus botryoides	22	2x	1.350	0.675	660.15	Almeida-Carvalho *et al.*(2017)
Genus *Psidium*						
Psidium guajava	22	2x	0.950	0.475	464.55	Machado-Marques *et al.*(2016); Coser *et al.* (2012)
Psidium guajava (purple)	18	2x	0.990	0.495	484.11	Souza *et al.*(2015)
Psidium guajava ("Paluma")	22	2x	1.020	0.510	498.78	Souza *et al.*(2015)
Psidium guajava (white cultivar)	22	2x	0.507	0.253	247.43	Coser *et al.* (2012)
Psidium guajava (red cultivar)	22	2x	0.551	0.275	268.95	Coser *et al.* (2012)
Psidium grandifolium var. *cinereum*	44	4x	1.280	0.640	625.92	Costa and Forni-Martins (2009)
Psidium grandifolium var. *argenteum*	44	4x	0.820	0.410	400.98	Costa and Forni-Martins (2009)
Psidium cattleianum	44	4x	1.053	0.526	514.42	Costa and Forni-Martins (2006b)
Psidium cattleianum	44	4x	1.990	0.995	973.11	Souza *et al.*(2015)
Psidium guineense	44	4x	2.020	1.010	987.78	Souza *et al.*(2015)
Psidium guineense	44	4x	1.850	0.925	904.65	Machado-Marques *et al.*(2016)
Psidium acutangulum	44	4x	1.167	0.583	570.17	Costa and Forni-Martins (2009)

*1pg DNA = 978 Mbp (Dolezel *et al.* 2003; Bennett *et al.* 2000).

burn and Birchler 2014). Additionally, with the current development of second and third generation sequencing techniques (NGS), information on genome size or C values are essential to establish appropriate experimental conditions, to effectively prepare genomic libraries and sequencing of complete genomes (Leitch and Leitch 2008). The C-values reported here may be used as a tool for genomic analysis in this species, which should benefit genetic improvement practices in this species.

Therefore, given the absence of information on ploidy level and nuclear DNA content in populations of *Psidium friedrichsthalianum*; we aimed to determine the chromosome number via fluorescent DAPI stain and flow cytometry to determine the nuclear DNA content of this tropical fruit in six populations of Costa Rica, its likely centre of origin.

MATERIALS AND METHODS

Sample collection

We analysed individuals from six populations of *P. friedrichsthalianum* in Costa Rica. Samples were collected from local small-scale plantations from different regions in the country (Table 2). Plantations were located at different elevations ranging from sea level to over 1500 m asl (metres above sea level). Samples were always taken from reproductive trees and care was taken to collect samples from individuals that were separated by at least 10 meters to avoid collecting possible genets.

Chromosomal count using DAPI stain

Chromosome counts were performed on pollen mother cells in meiotic metaphase. At least seven flower buds were collected from each of six populations; flower buds ranged between 0.7 cm and 0.8 cm in length. Flower buds were fixed in FAA solution (96% ethanol, 5% glacial acetic acid and 40% formaldehyde) for 24 hours. As suggested by Dyer (1979), flower buds were dissected to 3/4 of their final bud size. Anthers were placed on slides and subjected to mechanical disaggregation (macerating anthers with a thin spatula) adding occasional drops of acetic acid to prevent desiccation. Macerated anthers were stained with DAPI fluorochrome (Sigma-Aldrich, Ilinois, USA) and were incubated in the dark for 5 min. We used an epifluorescence microscope (Olympus BX50, Olympus Corporation, Tokyo, Japan) to visualize and photograph stained cells. Only cells that were in a state of meiotic metaphase were photographed. For each population at least five slides were evaluated and at least one cell was recorded in a state of meiotic metaphase. Finally, to facilitate chromosome counts, image color adjustments were performed with Adobe Photoshop CS82 (Adobe Systems, San Jose, CA) and the ImageJ software (US National Institutes of Health, 2007) was used to count chromosomes.

Flow cytometry estimates of DNA content

We used flow cytometry to estimate genome size on *P. friedrichsthalianum*. We collected fruits from at least 10 individuals per population. Seeds were manually extracted, washed with tap water and dried in open air for a week to remove moisture. After one week, seeds were sown in pots with vermiculite soil and placed in a greenhouse for 12 weeks until seedling emergence. Ten seedlings per population were analyzed in a BD FACSCalibur TM (Becton Dickinson, San Jose, CA, USA) flow cytometer. After initial parameter adjustment in the flow cytometry equipment, samples were prepared following the protocol by Dolezel *et al.* (2007) with some modifications. We used 1 mg of leaf sample from *Glycine max* as a reference (2C = 2.50 pg) (Dolezel *et al.* 2007) and 5 mg of leaf tissue from *P. friedrichsthalianum*. Leaves were

Table 2. Collection sites of "Costa Rican Guava" used to determine genome size and chromosomal number. **PPT**: mean annual precipitation (mm); **Samples CMF**: number of seedlings used for flow cytometry; **Samples NM**: number of samples used to determine chromosomes count.

Population name	Geographical coordinates		Altitude (m a.s.l.)	PPT (mm)	Temp (°C)	Samples CMF	Samples NM
Cervantes	09°53′28.3″N	83°47′24.0″W	1465	2500	24	10	5
Guápiles	10°13′42.1″N	83°46′06.3″W	262	4535	27	10	5
Tacacorí	10°03′07.3″N	84°12′52.3″W	952	2100	23	10	5
Ciruelas	09°59′05.7″N	84°15′26.3″W	910	1900	23	10	5
Batán	10°04′34.4″N	83°22′37.2″W	114	3567	28	10	5
Escazú	09°55′08.3″N	84°07′42.6″W	2428	1929	24	10	5

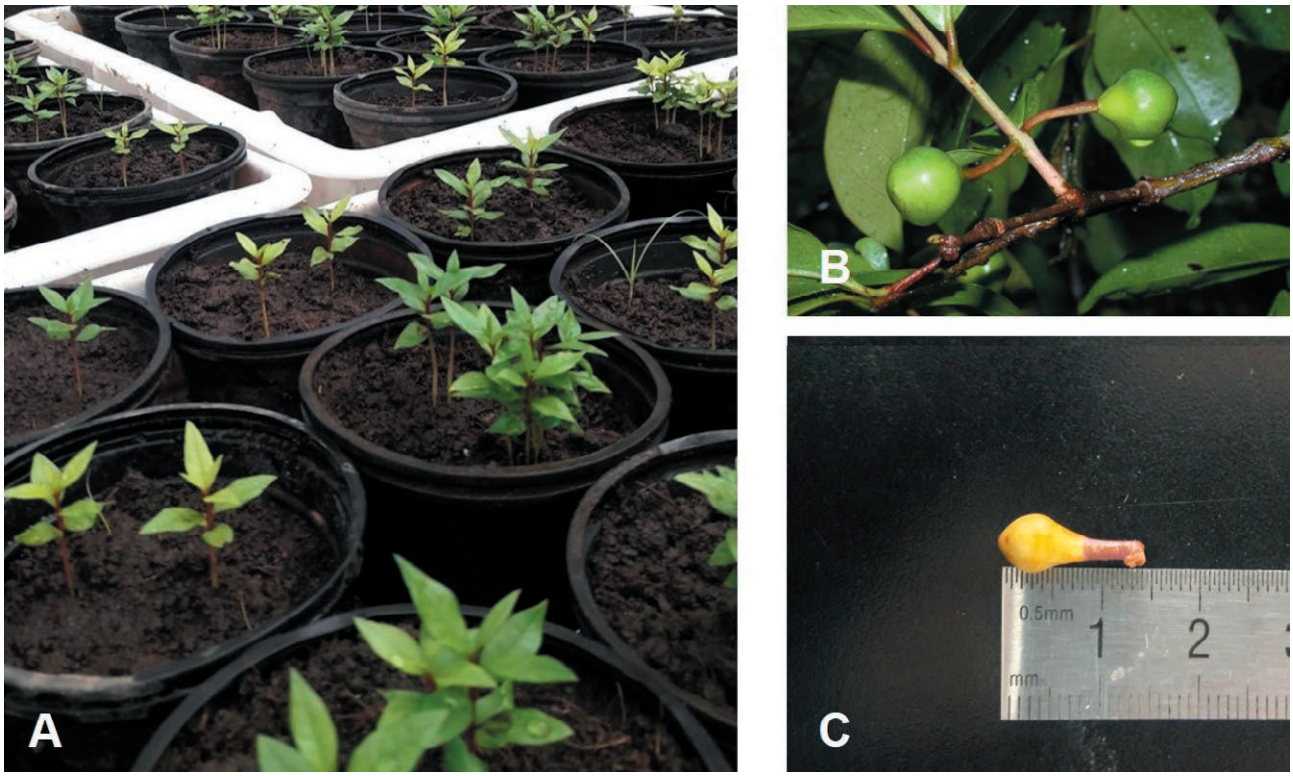

Figure 1. (a) Seedlings of *P. friedrichsthalianum* used to measure genome size by flow cytometry. (b and c) Flower buds of *P. friedrichsthalianum* used for cytogenetic observations.

placed in a petri dish on ice, then 1 ml of OTTO-I lysis buffer (0.1 M citric acid, 0.5% (vol / vol) Tween 20) supplemented with 2 mM dithiothreitol (DTT) was added to the leaf cutouts (Otto 1990). Subsequently, leaves were cut with a razor blade until homogenization. The extract was filtered through a 41µm Nylon mesh, onto a 2.0 ml microcentrifuge tube. The filtrate was centrifuged at 10,000 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in 100 µl of OTTO-I lysis buffer and incubated for 15 min at 4 ° C. After incubation and prior to analysis in a flow cytometer, 300 µl of OTTO-II buffer (0.4 M Na 2 HPO 4 · 12 H 2 O) (Otto 1990), 20 µl of propidium iodide (50 µg / ml) and 2 µl of RNase (50 µg / ml) were added to the mixture.

All measurements were based on the fluorescence of at least 5000 total events (total nuclei). We analyzed two independent replicas of each sample on different days and estimated an average nuclear DNA content. Mean fluorescence intensity (MFI), number of events per peak and variation coefficient were all calculated using the FCS Express 4 Flow Cytometry software (De Novo Software, Los Angeles, CA). Finally, the nuclear DNA content was calculated according to Dolezel *et al.* (2007) as follows:

$$A = \frac{(B \times C)}{D}$$

Where A = 2C (pg) nuclear DNA content concentration of *P. friedrichsthalianum*; B = Mean fluorescence intensity (MFI) of the G_0 / G_1 peak of *P. friedrichsthalianum*; C = 2C (pg) nuclear DNA content of the internal standard; D = MFI of the G0 / G1 peak of the internal standard. Genome size was estimated from DNA content as 1 picogram (pg) of DNA being equivalent to 978 megabase pairs (Mbp) (Bennett *et al.*2000; Dolezel *et al.* 2003). The 2C nuclear DNA content data of all individuals was compared among populations with a one-way ANOVA, followed by Tukey's test to determine individual differences ($p < 0.05$). Statistics were done using R 3.5.0 software (R Core Team 2018).

RESULTS AND DISCUSSION

We consistently found 11 bivalent chromosomes in all meiocytes from *Psidium friedrichsthalianum* (Figure 2) across all populations (Table 2). Taking into account that the basic chromosome number of the Myrtaceae

family is x = 11 (Atchison 1947; Raven 1975) we classified all Costa Rican samples of *P. friedrichsthalianum* as diploid (2n=2x=22). The diploid nature of the Costa Rican guava mirrors the results from Srivastava (1977), who similarly found a 2n = 2x = 22 diploid chromosome count in different genotypes of *Psidium friedrichsthalianum*. Costa and Forni-Martins (2006a, b, 2007) also described chromosome numbers for 50 species in the Myrtaceae, and found a predominance of 2n = 2x = 22 diploid species. Naitani and Srivastava (1965), Coser *et al.* (2012), Éder-Silva *et al.* (2007), and Souza *et al.* (2015), all found predominantly diploid species in the *Psidium* genus, such as in *Psidium chinense* and *Psidium guajava*.

Previous results reported *P. friedrichsthalianum* individuals with 2n=4x=44 and even 2n=6x=66 (Hirano 1967), suggesting that this species may have tetraploid and hexaploid members. These results clearly indicate that there may be variation in ploidy levels among populations of *P. freidrichsthalianum* in different areas. In contrast, our results show that at least in Costa Rica, cultivated populations are consistently diploid. This chromosomal uniformity may be the result of a common historical origin among populations. Alternatively, our results may also be a consequence of artificial selection by farmers who selected cytotypes with specific homogenous traits of interests such as fruit size of pulp content. Multiple cytotypes have also been found in other *Psidium* congeners, for example in *Psidium cattleyanum* the cytotypes 2n = 44, 66, 77 and 88 have been described (Costa and Forni-Martins 2006a; Costa 2009). Multiple cytotypes have been also found in populations of *Psidium guineense* and *Psidium guajava* (Srivastava 1977; Costa and Forni-Martins 2006a; Éder-Silva *et al.* 2007; Souza *et al.* 2015). Polyploidy is recognized as one of the main evolutionary forces in angiosperms (Soltis *et al.* 2015); and it is frequently associated with interspecific hybridization followed by chromosomal duplication to restore hybrid fertility (Soltis *et al.* 2009). Results from congeners suggests that *P. friedrichsthalianum* may also have the potential to create other cytotypes may represent important prospects for future breeding programs.

Our study found bivalent and univalent chromosomes in meiocytes of *P. freidrichsthalianum* (Figure 2a-2c). Chromosomes were also observed in a trivalent state (Figure 2d) and this is consistent with previous observations by Srivastava (1977) in this species. Univalent chromosomes are frequently observed in plants; these can arise through three different ways: (i) when a chromosome is not matched completely in zygotene stage; (ii) when paired bivalents separate in diplotene because robust chiasmata have not yet formed between them; (iii) due to premature disjunction of the bivalents during anaphase (Pires-Bione *et al.* 2000). The premature migration of univalent chromosomes to the poles during cell division is common in plants, giving rise to micronuclei (Pagliarini 1990; Pagliarini and Pereira 1992; Consolaro *et al.* 1996). Alternatively, univalent chromosomes may occasionally occur in plants due to environmental factors such as temperature fluctuations (Heilborn 1934; Katayama 1935). Some of our sites differ drastically in climatic conditions, however, further studies are needed to better understand the cytology of this species.

Our flow cytometry estimates were very consistent across all plant samples. Our coefficients of variation were all less than 5% (Table 3), which confirms that our suspensions had a sufficient number of stoichiometrically stained and intact nuclei. Additionally, DTT used in nuclei suspensions proved to be effective inhibiting cytosolic interfering compounds which resulted in clear histograms. DTT is commonly used in flow cytometry studies because of its broad antioxidant activity (Dolezel *et al.* 2007). In our study, DTT was very efficient because many woody species in the Myrtaceae, as is the case of *P. friedrichsthalianum*, contain abundant secondary metabolites that may interfere with DNA content staining (Loureiro *et al.*2006) (Ohri and Kumar 1986).

We determined a mean nuclear value of 2C = 1.960 ± 0.005 pg for *P. friedrichsthalianum*, equivalent to

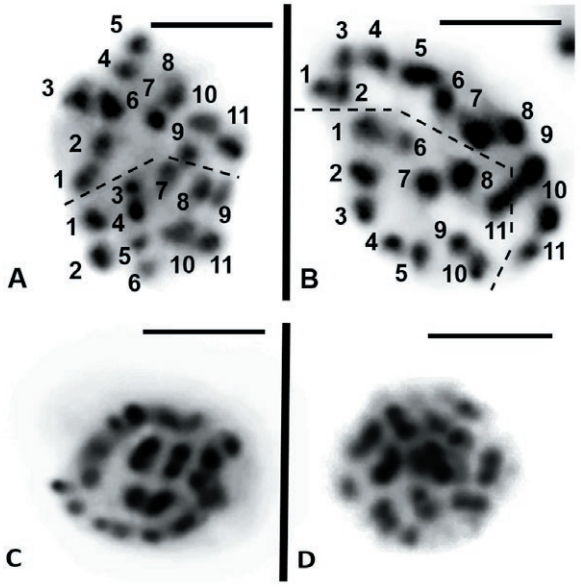

Figure 2. Bivalent chromosomes of *P. friedrichsthalianum* in meiotic metaphase, stained with DAPI, scale bar 10um. (a, b and c) Samples from the populations of Cervantes, Tacacorí and Escazú respectively, showing 11 bivalent chromosomes. (d) Image showing chromosomes in trivalent, bivalent and univalent states.

Table 3. Parameters obtained by flow cytometry to determine the genome size of *Psidium friedrichsthalianum*. **NE**: Number of events obtained; **CV**: Coefficient of variation obtained; **2C (pg)**: holoploid nuclear DNA content obtained; 1pg DNA = 978 Mbp (Dolezel *et al.* 2003; Bennett *et al.* 2000).

Species	NE	CV	1C (pg)	2C (pg)	Mbp
Psidium friedrichsthalianum	2452 ± 0.001	2.95 ± 0.007	0.980 ± 0.005	1.960 ± 0.005	1916.88
Glycine max (*standard*)	2756 ± 0.005	3.01 ± 0.005			

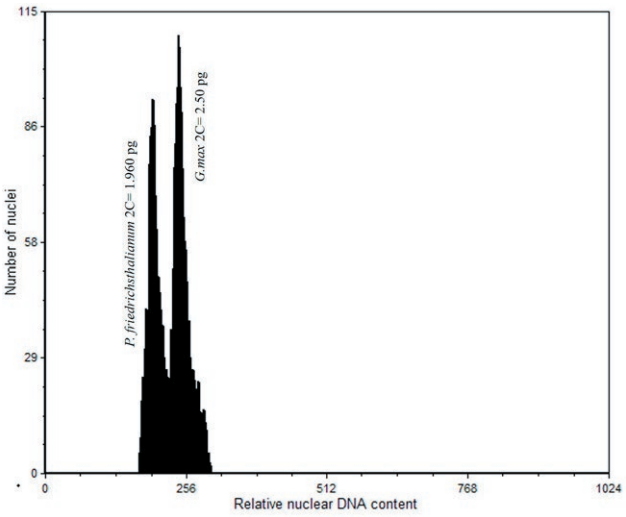

Figure 3. Relative fluorescence intensity (propidium iodide (PI)) histogram obtained after a simultaneous cytometric analysis of nuclei of reference standard (*Glycine max,* 2C=2.50 pg of DNA) and *Psidium friedrichsthalianum* (2C= 1.960 ± 0.005 pg).

1916.88 Mbp (Bennett *et al.* 2000) (Figure 3, Table 3). Nuclear DNA content did not statistically vary among all six populations (F=0.29; df=5; p = 0.917). Leitch *et al.* (1998) and Soltis *et al.* (2003) classified species with 1C ≤ 1.4 pg content as species with a very small genomes compared to other angiosperms. Therefore, given our 1C estimates (1C = 0.98 ± 0.005 pg) (Table 3) the Costa Rican guava should also be classified as a small genome species. Consistently, Machado-Marques *et al.* (2016) found that *Psidium guajava* and *Psidium guineense*, also have very small genomes as 1C = 0.475 pg and 1C = 0.925 pg, respectively (Table 1). Almeida- Carvalho *et al.* (2017) determined that 25 species of the genus *Eucalyptus* (Myrtaceae), all had 1C values between 1C=0.40 pg and 1C=0.75 pg which may indicate that the Myrtaceae family may typically contain species with smaller genomes. On the other hand, our 2C estimates (2C =1.960 ± 0.005 pg) are within the range described by Souza *et al.* (2015), who also used flow cytometry on different species of *Psidium* and found 2C values that ranged between 2C=0.99 pg and 2C=5.48 pg. However our estimates are significantly higher than those found for different varieties of *Psidium guajava*; for example, Coser *et al.* (2012) found 2C = 0.507 pg for the white varieties, and 2C = 0.551 pg or 2C = 0.950 pg for the red varieties; while Souza *et al.* (2015) found 2C = 0.990 pg and 2C = 1.020 pg in purple and "Paluma" varieties respectively (Table 1). These differences in genome size may be due to (i) natural or bred adaptations of these species to different environmental conditions (Cavallini and Natali 1990), for example, to new cultivation environments; (ii) hybridization events, or (iii) changes in repetitive DNA sequences (Martel *et al.*1997). Several authors have suggested that transposable elements (TE) may be important in the evolution of genome sizes in plants (Wang *et al.* 2016; Wendel *et al.* 2016; Zhao *et al.* 2016). For example, Almeida-Carvalho *et al.* (2017) compared the genome size of two *Eucalyptus* species, *E. botryoides* (2C=1.350 pg) and *E. microcorys* (2C=1.040 pg); and found big differences in genome size between them, although both had the same chromosome number (2n = 2x = 22) (Table 1). They argued that variations in 2C values in these *Eucalyptus* were caused by chromosome rearrangement and possibly TE elements.

Therefore, our results on ploidy level and genome size of *P. friedrichsthalianum*, contribute to the cytogenetic characterization of this economically important fruit species. This information may be used to design regional conservation strategies that preserve local genetic resources. Flow cytometry may be used to assess ploidy level in *in vitro* propagated plants (Ochatt *et al.* 2011), to screen for plants with higher ploidy levels, which may have new features of economic interest such as increased fruit size, or better juicing capabilities. Additionally, results from our study could aid the taxonomic definition of *P. friedrichsthalianum* species and the understanding of phylogenetic relationships among other members in the genus *Psidium*.

CONCLUSIONS

Populations of *Psidium friedrichsthalianum* from six different regions of Costa Rica, the likely centre of origin

of this species, have a chromosome number equal to 2n = 2x = 22, indicating that cultivated populations in Costa Rica, are all consistently diploid. Furthermore, these populations have an average 2C nuclear DNA content of 1.960 ± 0.005 pg. The uniformity found across populations in terms of chromosomal number and nuclear DNA content, suggests a common origin among them.

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GEOLOCATION DATA

Geolocation data is found on Table 2.

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Karyotype analysis of *Trichogramma embryophagum* Htg. (Hymenoptera: Trichogrammatidae) using a new method and estimate its karyotype symmetry

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Abstract. Different methods of chromosome preparation for insect are now being used across the world. Well-spread chromosomes with explicit morphology, in addition to no cell wall debris are required for karyotype investigations. Cytogenetic knowledge in *Trichogramma* is extremely limited. In the article, chromosome characters, karyotype and monoploid ideogram of *Trichogramma embryophagum* Htg. (Hymenoptera: Trichogrammatidae) were evaluated using a new method. Also, karyotype symmetry/asymmetry of this species was calculated for the first time as one of the *Trichogramma* species. Our results showed that the diploid chromosome number of the wasp was $2n = 10$. The karyotype formula was 6m + 4a. The symmetry/asymmetry index value was 1.8. The new method resulted in higher quality metaphase plates spread and provided an ideal karyomorphology for this parasitoid which has small chromosomes.

Keywords: ideogram, karyotype, 8-hydroxyquinoline, symmetric karyotype.

INTRODUCTION

A high-quality chromosome preparation is critical for cytogenetic studies of Hymenoptera parasitoids. In the majority of parasitoid species, complications are caused by the minute size of the insect (Baldanza *et al.* 1993), the small size of their chromosomes (Paladino *et al.* 2013), and low number of chromosomes, $2n = 6$ found in *Brachymeria intermedia* (Chalcididae) and *Encarsia protransvena* (Aphelinidae) (Hung 1986; Baldanza *et al.* 1999) which make their cytogenetic studies limited. Although modern molecular techniques such as Fluorescent *in situ* Hybridization (FISH) have already taken been on an important role (Yalcin and Kulduk 2018), they have never been able to be a complement replacement for classical methods. Some disadvantages including being costly and difficult (due to requiring probes and fluo-

rescent microscope), requiring highly-skilled cytogeneticists, and the inability to reveal chromosome inversions (Di- Nizo *et al.* 2017) have caused cytogenetics to show a significant tendency towards using classical methods.

Squash is a well-known and oldest method for flattening metaphase plate in insects (Kocak and Okutaner 2017). However, difficulty in achieving well-spread, distorted, and stretched chromosome are the main disadvantages of this method (Chirino *et al.* 2014; Kocak and Okutaner 2017). During squashing, producing well-spread plates are often not desirable due to the concentration of cells in a small area. Over time, spread (hot-plate spread) method conducted by various researchers flourished (Bressa *et al.* 2009; Sadilek *et al.* 2016). Its major disadvantage is the attendance of cellular debris around the chromosomes (Chirino *et al.* 2014), making their morphology unpleasant. Considering the mentioned constraints, there is always a need for a simple and efficient method for the preparation of metaphase plates in parasitoids.

Despite the vast amount of research on insect karyotype, only about 500 species of parasitoid wasps have available data (Gokhman 2009). Among these, only more than 10 are related to *Trichogramma*. So far, 239 species of *Trichogramma* have been reported throughout the world (Khan and Yousuf 2017). There are eleven species of this parasitoid in Iran (Nazeri *et al.* 2015) that *Trichogramma embryophagum* Htg. is one of them. *T. embryophagum* distributed in some of regions of Iran, including the provinces of West and East Azerbaijan, Yazd, Khorasan (Poorjavad *et al.* 2011). Cytogenetic studies and karyotype investigation have not yet been done on this species. Because of the substantial position that *Trichogramma* occupy in the biological control programs (Parra 2009), investigations on their chromosome details have provided precious information for the understanding various perspectives such as mechanisms of sex determination, evaluation of sex chromosome, existence of accessory chromosomes and phylogenetic relationships.

An evaluation of existing literature shows that different methods was used in karyotype studies of *Trichogramma*. Studies about *Trichogramma* were derived from routine chromosomal preparation methods, including the use of various percentages of colchicine as pretreatment, staining by different colors, and then squashed (Hung 1982; Liu and Xiong 1998). Interestingly, no banding staining on the *Trichogramma* species has ever been reported. Molecular cytogenetic including Florescence *in situ* hybridization (FISH) has been applied for only the two species of *T. kaykai* Pinto & Stouthamer (van Vugt *et al.* 2005) and *T. pretiosum* Riley (Gokhman *et al.* 2017). It should be noted that the problems described for each of these methods also exist for *Trichogramma* karyotype studies. The minute size and lifestyle of *Trichogramma* species also intensify these problems (Manickavasagam 1991; Hung 1982). In most studies, only the haploid number of these species has been raised, and the chromosomal details including the length of the arms that could provide many comparisons were not possible (Hung 1982; Laurent *et al.* 1998). One parameter that can be obtained from the chromosomal information is karyotype symmetry/asymmetry (Peruzzi and Eroğlu 2013). Karyotype symmetry/asymmetry is obtained based on the availability of chromosome detail, including relative chromosome size and position of centromere. Until now, this parameter which can help in the evaluation of enter- species relationship seldom has calculated in class of insect. In the cytogenetic studies, species that are more similar in the terms of chromosomal parameters such as karyotype symmetry/asymmetry will have more affinity.

In this research, the karyotype, ideogram, and chromosomal detail of *T. embryophagum* were investigated for the first time with the introduction of a new method considering all steps of Hymenoptera parasitoids chromosome preparation. These chromosome analyses were complemented by an estimation of its karyotype symmetry as one of the species of Trichogrammatidae.

MATERIAL AND METHOD

Insect

T. embryophagum Htg. individuals originated from parasitized eggs of carob moth, *Ectomyelois ceratoniae*, (Zeller) (Lepidoptera: Pyralidae), were collected on pomegranate in the center of Iran (Yazd Region, 32.1006° N, 54.4342° E). The wasps were reared on *Ephestia kuehniella* Zeller (Lepidoptera; Pyralidae) eggs in a climate-controlled chamber at 25 ± 1 °C, 70 ± 5% relative humidity, L16: D8 photoperiod. *E. kuehniella* eggs were obtained from a culture maintained in the Biosystematics Laboratory at The University of Tehran, Karaj, Iran.

Preparation chromosome

Sample from egg host were Dissected out in physiological solution for *Ephestia* (Glaser, 1917, cited by Lockwood 1961). Two kinds of pre-treatment were used, including hypotonic and other is combined it. First, samples were transferred into a drop of 0.075 M KCl for 8 minutes [0.075 M KCl: 0.5592 g KCl/100 ml redistill.

H_2O] on a shaker at 20 °C and 30 rpm. After wise, the samples were excised and pretreated in solution mixture of 8-hydroxiquinoline (0.002 w.v): colchicine (0.05 w.v) contains low concentration of dimethyl sulfoxide (DMSO) at about 4° C for 30 minutes on shaker 30 rpm; and were washed in hypotonic solution (NaCl 0.9% + KCl 0.042% + $CaCl_2$ 0.025% in distilled water) for 3 times. Then they were Fix in freshly prepared Carnoy`s fixative (6:3:1 - ethanol: chloroform: acetic acid) for 20-30 minutes. They were Transferred on a clean slide into a drop (5-10µl) of 60% acetic acid and their head was cut off from other parts of the body with tungsten needles, after a while, 5-10µl of 60 % acetic acid was added again. The slides were put on a heating plate at 45 °C until the acetic acid almost evaporates. Water was removed bypassing the slides through an ethanol series (70 %, 80 %, 96 %; for 30 seconds each of it, respectively) and the samples were let air-dried. The slides were stained immediately with 5 % Giemsa in phosphate buffer (pH 6.8).

Microscopic photograph and analysis

The chromosome slides were examined under Olympus BX53 microscope and chromosomes were photographed using an Olympus DP72 camera. Chromosome measurements were made on 10 metaphase plates by application of KaryoType software (Altınordu *et al.* 2016). Arm rations, average lengths, and centromeric index were calculated and then were classified according to Levan *et al.* (1964) considering their centromere position.

The karyotype symmetry/asymmetry was calculated. The formula for symmetry/asymmetry index is given below.

$S/A_I = (1 \times M) + (2 \times SM) + (3 \times A) + (4 \times T) / 2n$ (Eroğlu 2015).

Eroğlu (2015) reported the new classification model from full symmetric to full asymmetric with five different types. The chromosomes, are all metacentric, form full symmetric karyotype; unlike those, are all telocentric, form full asymmetric karyotype.

RESULT

The karyotype of *T. embryophagum* Htg. contained three pairs of large metacentric and two considerably smaller pairs of acrocentric chromosomes. Two of the metacentric chromosomes were of similar size whereas the third pair was close to submetacentric. Thus, the diploid chromosome number was $2n = 10$, and the karyotype formula was 6m + 4a (Figure 1a, b). The detailed chromosomal data are given in Table 1, and monoploid ideogram is given in Figure 2.

In *T. embryophagum*, haploid chromosome length and mean chromosome length are 6.38 and 1.28 µm. The rates of relative length and centromeric index range from 9.40 to 28.06 and from 18.33 to 48.04, respectively. The symmetry/asymmetry index value is 1.8.

DISCUSSION

In this research, the main issues were to have a regular cell layer without overlapping and the absence of qualitative damages to the morphology of chromosomes while allowing the possible spread of chromo-

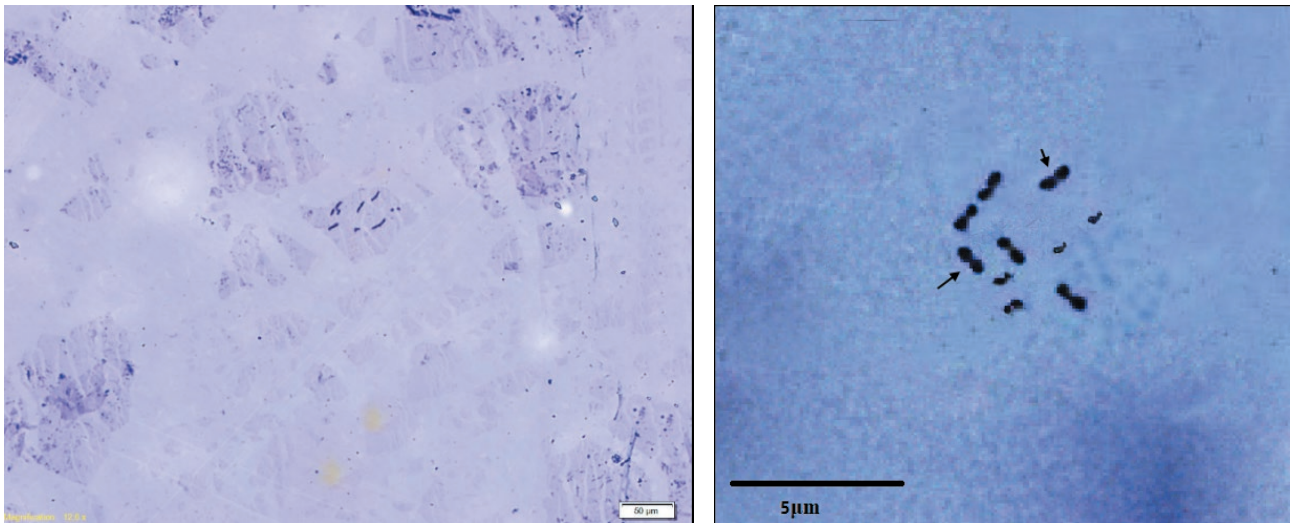

Figure 1. a, b) Mitotic metaphase plate of *T. embryophagum*. Giemsa staining. Magnification 100x.

Table 1. The detailed measurement data of chromosome pairs of *Trichogramma embryophagum*.

Pair	L (µm)	S (µm)	L + S (µm)	RL (%)	L / S	CI (%)	Type
1	0.93	0.86	1.79	28.06	1.08	48.04	m
2	0.91	0.73	1.64	25.71	1.25	44.51	m
3	1.02	0.61	1.63	25.55	1.67	37.42	m
4	0.58	0.14	0.72	11.29	4.14	19.44	a
5	0.49	0.11	0.60	9.40	4.45	18.33	a

Abbreviations: long arm length (L), short arm length (S), total chromosome length (L + S), relative length (RL), arm ratio (L/S), centromeric index (CI), metacentric (m), acrocentric (a).

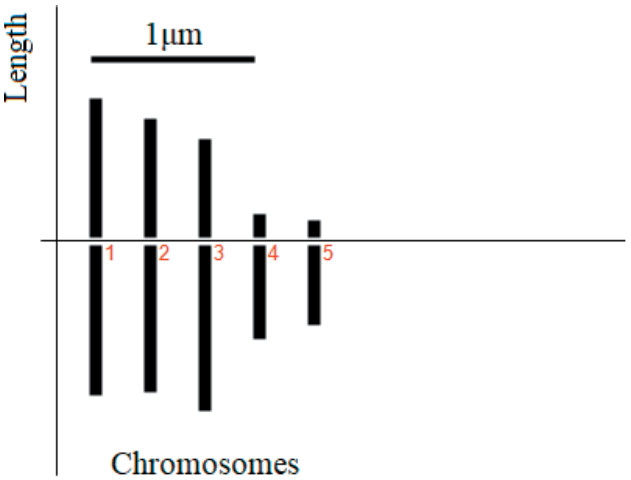

Figure 2. Ideogram of *T. embryophagum*. Chromosomes are numbered from the longest (1) to shortest (5).

somes within the cell. The method explained here has aimed at achieving both goals. The chromosome structure had a high resolution, which is related to how the chromosomes was prepared. The process of karyotyping is completed through some steps such as pretreatment, fixation and staining. On both sides of the cell, outside and inside, the concentration of water is the same, and Potassium and sodium ions are the most osmotically solutions inside and outside of the cell, respectively (Leaf 1973). In this case, the cell is in an isotonic state. The KCl changed the osmosis of the cell. Pre-treatment by KCl affects both cellular swelling and better chromosomes spread. About KCl, hypotonic rate and duration of treatment are important. Rupturing the cell membrane and the damages of chromosome (Earley 1975) can be caused by the lack of attention to these cases. Our result indicated that the hypotonic rate and duration of KCl reported here overcome protoplast damage. Sadilek *et al.* (2016) stated that KCl causes the osmosis of the cell due to receiving additional water that resulted in a larger cell. This process effects more identified of the chromosome. The next step after KCl was using combined pretreatment. Colchicine is a substance that affects the metaphase stage of the dividing cell. Guo *et al.* (2018) stated that colchicine increased the yield of the metaphase plate. The 8-hydroxyquinoline is effective in prolonging the metaphase and further compression of the chromosomes. It is noteworthy that, until the present time, this substance has not been used as pre-treatment in insect karyotype studies but has been used extensively in plant karyotype. This substance can maintain the shape of the chromosome during the preparation process. This feature has been used in some plant studies (Fernandez *et al.* 2009; Zarifi and Güloğlu 2016). We found that 8-hydroxyquinoline prevented the distortion and stretch of the chromosome, which are the main drawbacks of squash and spreading methods. Use of colchicine alone showed each chromosome clumped exceedingly and their centromeres were not distinctly in our pre-tests while using 8-hydroxyquinoline alone, the metaphase plates were few. As a result, a combination of two substances as pretreatment improved chromosome spreading. Ma *et al.* (1996) reported that the effects of these materials together make this as improvement whereas colchicine depolymerized microtubules, 8-hydroxyquinoline decreased the rate of progression among mitotic stages and also resulted in a disorderliness in chromosome movement. In combined pretreatment, dimethyl sulfoxide (DMSO) affected the better penetration of compounds of pretreatment into the cell. DMSO has been reported as an effective penetration enhancer (Gurtovenko and Anwar 2007; Williams and Barry 2012). Notman *et al.* (2006) stated that DMSO induces pores in the membrane. Another study indicated that DMSO concentration is critical (Gurtovenko and Anwar 2007). We used a low concentration of DMSO in our method. In this concentration, DMSO induces membrane thinning (Gurtovenko and Anwar 2007).The approach used in the present study focused on providing a quick and easy method for those insects which have small chromosomes, especially *Trichogramma* wasps. The advantages of the new method are as follows:

1. In the present method, the chromosomes are readily accessible through morphological features, including the centromere position. According to this, the evaluation of all of the parameters such as karyotype symmetry/asymmetry can be available.

2. Higher quality metaphase plates spread which is achieved through the use of a combined pre-treatment which its results are an ideal karyomorphology.

3. Providing a single layer of cell and decreasing the overlapping cells, therefore, a better selection of metaphase plates is possible.

The Trichogrammatidae species were less likely to be karyotypically studies in comparison with other parasitoid families. The karyotype *T. embryophagum* Htg. consists of 10 chromosomes (n= 5, $2n$ = 10). Members of this family showed 10 chromosomes previously published (Hung 1982; Van Vugt *et al.* 2003; Gokhman *et al.* 2017), although there is an exception, *T. kaykai*, which has been diagnosed with one B chromosome that is termed *psr* (from Paternal Sex Ratio) (Stouthamer *et al.* 2001). The metaphase images indicated that chromosomes are metacentric (first, second and third chromosome pairs) and acrocentric (fourth and fifth chromosome pairs). According to these chromosome types, the S/A_I value is 1.8, and the karyotype is symmetric type in *T. embryophagum*. Karyotype symmetry/asymmetry is one of the cheapest and most popular parameters that can be obtained from cytogenetic studies (Peruzzi and Eroğlu 2013). The symmetric karyotype is characterized by an excess of metacentric, submetacentric chromosomes. In *T. embryophagum* due to two pairs of acrocentric chromosomes, S/A_I value is close to 2.0 (between symmetric and asymmetric). Gokhman (2009) expressed that two trends have occurred in evolution of karyotype in parasite Hymenoptera. First one is decreasing in chromosome number, and the other one is karyotype dissymmetrisation, which happened as a result of the proportion acrocentric in a chromosome set. Despite the widespread use of various methods that are adopted to calculate the symmetry/asymmetry karyotype in plants, this parameter has not been taken into consideration in other organisms (Eroğlu 2015). So far, karyotype symmetry/asymmetry has been seldom calculated in the class of insects and other species of parasitoids. Our result showed for the first time that in one of the species of *Trichogramma* wasps, the karyotype is symmetric type according to a new method. Karyotype symmetry/asymmetry is applied on one hand to determination evolutionary relationship, and on the other hand, to compare different levels of the taxonomy (Eroğlu 2015). In the case of Trichogrammatidae, information obtained due to the lack of other information's in this case is not possible to assess the issue mentioned above in this time but our results can be used as a basis for future studies in the above fields.

In conclusion, the described method is cost-effective and technically easier to make for the resolution of chromosome details. The morphological characteristics of each chromosome are better observed. Also, no special equipment is required as compared to molecular methods. According to the described method preparation of chromosome detail, karyotype and also ideogram of *T. embryophagum* were provided for the first time. We adduce as other species of *Trichogramma* are also evaluated in which the diploid number is $2n$ = 10. The karyotype is a symmetric type.

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Assessment of anti-cytotoxic, anti-genotoxic and antioxidant potentials of Bulgarian *Rosa alba* L. essential oil

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Abstract. Bulgarian *Rosa alba* L. essential oil is widely used in perfumery, cosmetics and pharmacy. The scarce data about its cytotoxic/genotoxic effect and anti-cytotoxic/anti-genotoxic potential gave us a reason to set our aim: i) to study its cytotoxic/genotoxic activities, iii) to explore its cytoprotective/genoprotective potential against the experimental mutagen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in two experimental test-systems - barley and human lymphocytes using appropriate endpoints and iii) to assess its antioxidant properties. Findings about chemical composition of rose essential oil would help us to explain its activities. Chromatogaphic profile of rose essential oil was obtained by Gas Chromatography-Mass Spectrometry and quantificaton of particular constituents was done with a Gas Chromatography-FID system. Superoxide anion radical scavenging, DPPH inhibition and iron ion chelating activity were used to study a possible antioxidant potential of the rose oil. Its defense potential was investigated by induction of chromosome aberrations and micronuclei in both test-systems. Cytogenetic analysis showed a low cytotoxic effect in both test-systems and no high genotoxic effect in human lymphocytes *in vitro* in a dose-dependent manner. Rose oil possessed a well-expressed anti-cytotoxic/anti-genotoxic potential against MNNG manifested by decreasing both of chromosome aberrations and micronuclei regardless of the experimental schemes used. A well-expressed concentration-depended free radical scavenging activity of the essential oil was obtained. Current data suggest a promising ethnopharmacological potential of Bulgarian white rose essential oil.

Keywords: *Rosa alba* L. essential oil, rose oil components, genotoxicity, anti-cytotoxicity, anti-genotoxicity, antioxidant effect.

INTRODUCTION

As a general rule, living organisms exist in conditions of continuous attack by various environmental pollutants such as alkylating agents, oxidative stress inducers, etc. As a result serious alteration in the main hereditary molecule DNA could be induced. There is a constant need to obtain products, which could decrease or eliminate the harmful effects of the genotoxins. Plants are known as a rich source of various bioactive natural compounds, which are widely used in various areas of human life. A cursory look at the literature cited in relation to plants' essential oils in recent years indicates that there is a growing interest in evaluation of the biological activities of various extracts of essential plants, their antimutagenic and antigenotoxic potential (Blasiak *et al.* 2002; Mezzoug *et al.* 2007; Bakkali *et al.* 2008; Vicuña *et al.* 2010; Siddique *et al.* 2010; Arumugam *et al.* 2010; Leffa *et al.* 2012; Gokbulut *et al.* 2013; Madrigal-Santillán *et al.* 2013; Oyeyemia and Bakare 2013; Reddy and Devi 2014; Shohayeb *et al.* 2014; Laribi *et al.* 2015), and presuming their role in the prevention of degenerative diseases and other human ailments including cancer (Hajhashemi *et al.* 2002; Raut and Karuppayil 2014; Horváth and Ács 2015). The genus Rosa is one of the largest and most important aromatic and medicinal genera of the Rosaceae family. Numerous rose phytocomplexes, including essential oils isolated from *Rosa damascena* Mill., *Rosa x centifolia* L., *Rosa gallica* L., *Rosa alba* L. and *Rosa rugosa* Thunb. have been identified and used for therapeutic purposes as well (Rangaha 2001; Degraf 2003; Moein *et al.* 2010). The rose extracts help in the reduction of thirst, healing of old cough, special complaints of women, abdominal and chest pain, digestive problems and show skin health effects (Tabrizi *et al.* 2003; Boskabady *et al.* 2006; EMA/HMPC 2013). The rose essential oils and by-products of *Rosa damascena*, from Shafaa, Taif, Saudi Arabia (Shohayeb *et al.* 2014) and from Amman and Ajloun areas, Jordan (Talib and Mahasneh 2010) have antimicrobial activity against various Gram-positive, Gram-negative, acid-fast bacteria and fungi. Absolute oil of *Rosa damascena trigintipetala* Dieck has an antimutagenic activity against mitomycin C in normal human blood lymphocytes (Hagag *et al.* 2014). Methanolic and aqueous extracts of *Rosa damascena* white variety from Iran show better anti-radical activity than some synthetic antioxidants (Kashani *et al.* 2011). Unfortunately, little is known about single- and repeat-dose cytotoxicity, genotoxicity, carcinogenicity, reproductive and developmental toxicity, local tolerance or other special studies of preparations from rosae flos in animals and humans according to current state-of-the-art standards (Hagag *et al.* 2014).

Rosa alba L. is the second most important plant for Bulgarian rose production. Kovatcheva *et al.* (2011) and Dobreva (2010) demonstrated that Bulgarian *Rosa alba* L. has a similar oil composition but some of compounds are with lower content to that of *Rosa damascena* Mill. collected from Bulgaria. *Rosa alba* L. essential oil, known as Bulgarian rose oil of white rose, has been defined as "original, exclusively fine, only suitable for the highest perfumery" (Degraf 2003). Fukada *et al.* (2011) found that in an experimental model of acute stress in rats, inhalation with *Rosa alba* L. essential oil (supplied by Kanebo Cosmetics) lowered corticosterone levels almost twice. Water extract of calyces of *Rosa alba* from India might be a useful memory restorative agent in the treatment of cognitive disorders (Naikwade *et al.* 2009). Our previous study indicated well-expressed antimicrobial activities of Bulgarian *Rosa alba* L. essential oil (Mileva *et al.* 2014). Insufficient data exist about cytotoxic/genotoxic effect and anti-cytotoxic/anti-genotoxic potential of essential oil from Bulgarian *Rosa alba* L. This gave us a reason to set our aim in the present paper: i) to study cytotoxic and genotoxic activities of Bulgarian *Rosa alba* L. essential oil, ii) to explore its cytoprotective and genoprotective potential against well known experimental mutagen – alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in two types of widely used experimental test-systems (higher plant and human lymphocytes *in vitro*) using appropriate for this purpose endpoints, and iii) to assess its antioxidant properties. Phytochemical analysis of rose essential oil would help us to explain its cytotoxic/genotoxic and anti-cytotoxic/anti-genotoxic effects.

MATERIAL AND METHODS

Chemicals Used

All chemicals, standards and solvents used for analysis of rose essential oil (GC-MS, GC-FID), methods for testing of the antioxidant activity and cytogenetic analysis were of high purity (>99%). Tetracosane [646-31-1] GA14075, EC 2114745, free puriss. p.a. > 99.5 % (GC) used as a reference compound in Predicted Relative Response Factors calculations was purchased from Fluka, USA. Nitroblue tetrazolium (NBT), xanthine, α-tocopherol, butylated hydroxytoluene (BHT), ascorbic acid, ferrous chloride, ferrozine, EDTA, dimetylsulfoxyde (DMSO) used for examination of antioxidant properties and the chemicals used for cell cultivation and cytogenetical analysis (RPMI 1640, bovine serum, phytohaemagglutinin PHA, Giemsa) were purchased from Sigma-Aldrich, Germany. α-bromonaphthaline,

colchicine, KCL were purchased from Merck, Germany, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) from Fluka – AG, Switzerland and Schiff's reagent from Riedel-De Haen AG, Germany.

Plant material and distillation of Rosa alba L. essential oil

Fresh flowers of *Rosa alba* L., from the experimental field of the Institute of Rose and Essential Oil Plants (IREOP), in Kazanlak, Bulgaria were used as raw material in the study. The flowers were picked up in the mornings in May/June 2009, from 8 to 10 am, in a phase of semi-blooming – blooming. Roses were distilled immediately by water-steam distillation using IREOP's semi-industrial processing line. Process parameters of the distillation were as follows: a raw material for charge – 10 kg; hydro module 1:4, rate of 8-10% and duration – 150 min. The aromatic water was re-distilled in the same apparatus. The essential oil, obtained of each charge is the sum of primary and secondary oil in their natural ratio. Total oil is a mixture of distillates collected over 15 days – the time of the collection campaign of *Rosa alba* L. for 2009. Finally, it was dried with sodium sulfate (Merck, Germany), filtered and stored at 4 °C for further use.

The concentration of the main compounds as well as the physicochemical parameters and characteristics of the rose essential oil are controlled through implementation of national (BDS ISO9842:2006) and international Standards (ISO 9842:2003, www.iso.org) only for oil obtained from *Rosa damascena* Mill. So, in our study we used the essential oil from *Rosa damascena* Mill. cultivated in Bulgaria, Kazanlak as one of the controls in the tests for antioxidant activities.

Gas Chromatography-Mass Spectrometry (GC-MS)

Chromatogaphic profile of rose essential oil was obtained by Gas Chromatography-Mass Spectrometry. GC-MS analysis was carried out on a HP 6890 "PLUS" gas chromatograph interfaced with a 5975 mass selective detector. Separations were performed using a HP-5MS silica-fused capillary column – 30 m × 0.25 mm coated with 0.25 μm film of (5%-phenyl)- methylpolysiloxane as the stationary phase (Agilent Technologies, USA). The flow rate of carrier gas (helium) was maintained at 0.8 ml/min. The injector and the transfer line temperature were kept at 250 and 300 °C respectively. The oven temperature program used was 60 °C for 2 min then 3 °C/min to 300 °C for 8 min, total run time - 90 min. The injections were carried out in a split mode with a split ratio of 25:1. The mass spectrometer was scanned from 30 to 550 m/z. The injection volume was 1 μl.

The quantitative analysis was carried out on a HP 5890 "SERIES II" gas chromatograph equipped with a FID detection system. We analysed the same sample and the separation was performed at the same chromatographic conditions, column, carrier gas and temperature program. GC-FID – eluted constituents were identified on the basis of a Kovats Retention Index (RI), determined with reference to a homologous series of *n*-alkanes (C10-C28), under identical experimental conditions. GC-MS – eluted constituents were identified using MS Library search (NIST version 2.1), as well as by comparison with the fragmentation pattern of the mass spectra with data published in the literature (Adams 2007). For each identified constituent, from GC-MS analysis was obtained its Kovats RI. Every particular value for these indices was confirmed by the literature. The differences between the measured and published Kovats RI exceeding 10 units were not reported, except these for *n*-alcanes and a few unsaturated hydrocarbons. The percentage compositions of *Rosa alba* L. essential oil were determined from the GC-FID peak's areas corrected with Predicted Relative Response Factors for the constituents calcutated by formula given by Tissot *et al.* (2012).

Examination of antioxidant properties

Superoxide scavenging properties

The generation of superoxide anion radical $O_2^{\bullet -}$ in the model system xanthine-xanthine oxidase (XO) and the changes occurring upon the *Rosa alba* L. oil effect were investigated by the nitroblue tetrazolium (NBT) test. The detailed procedure was described elsewhere (Mileva *et al.* 2000). Briefly, the spectrophotometric registration of superoxide was carried out measuring the amount of formazan generated by $O_2^{\bullet -}$ induced reduction of NBT. The investigated samples of a volume 1 ml PBS contained: 1 mmol/l xanthine, 2.10^{-3} IU XO, 0.04 mmol/1 NBT, as well as oil at concentrations from 0 to 100 μg/ml. Samples were incubated at 37 °C and the amount of the formed formazan was measured by absorption at 560 nm. The time of incubation was selected so that the absorption for the controls was 0.2. The decrease of absorbance in the presence of oil indicated the consumption of superoxide anion in the reaction mixture. Data were calculated in percentage as spectrophotometric scavenger index (SpSI) - the ratio of the absorption at 560 nm for the sample with oil, and the same absorption for the controls (without oil). The scavenger activity of oil was compared with that of α-tocopherol – commonly used as superoxide radical scavenger.

DPPH Test

Hydrogen atoms and electron-donating potential of essential oils were measured from the bleaching of the purple-colored ethanol solution of DPPH (Sigma-Aldrich, Germany). All compounds were dissolved in ethanol to a concentration of 100 mg/ml stock solutions for the follow dilutions. DPPH assay was performed as follows: freshly prepared ethanolic solution of DPPH (100 mM) was incubated with tested substances in the concentration of 10 to 100 μg/ml, and the absorbance (A) monitored spectrophotometrically at 517 nm after 30 min incubation in dark at room temperature. Inhibition of DPPH in percentage (DPPH inhibition, %) was calculated as given below:

DPPH inhibition (%) = [(A control - A sample)/(A control)] X 100

BHT and ascorbic acid served as positive controls. Each experiment was carried out in triplicate and data were presented as a mean of the three values (Singh *et al.* 2008).

Iron binding capacity of essential oil

Metal chelating activity of *Rosa alba* L. essential oil on ferrous ions was determined according to the method of Decker and Welch (1990). The percentage of inhibition of ferrozine - Fe^{2+} complex formation was calculated using the formula:

Chelating activity (%) = [(A control – A sample)/A control] × 100

Where A control is the absorbance of the ferrozine - Fe^{2+} complex, and A sample is the absorbance of essential oil. As a positive control EDTA was used.

All results for antioxidant properties are presented as mean ± SD, and compared against routinely used reference positive controls. The data were collected from three independent experiments with three parallel measurements for each experiment.

Analysis of cytotoxic/anti-cytotoxic and genotoxic/anti-genotoxic effects of Rosa alba L. essential oil

Rose essential oil and chemicals preparation

Rose essential oil was dissolved in 1% dimetylsulfoxyde (DMSO). A standard well-known experimental mutagen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) 50 μg/ml was used as DNA damage agent in the chromosome aberrations and micronucleus assays. MNNG was dissolved in bidistilled water.

Test-systems

Two types of experimental test-systems were used – *Hordeum vulgare* (barley) and human lymphocytes *in vitro*. Barley seed meristems and human lymphocyte cultures preparation as well as the schemes of treatment are given below.

Hordeum vulgare (barley) meristem cells as a test-system. Seeds of reconstructed karyotype of *Hordeum vulgare* MK14/2034, 2n = 14 (Künzel and Nicoloff 1979) presoaked for 1 hour in tap water were germinated for 18h in Petri dishes on moist filter paper at 24°C. Well-synchronized seeds with a root meristem size of 1-2 mm were selected for further treatment.

Experimental designs used for cytogenetic analysis. Three types of experimental schemes were applied. First to assess cytotoxic/genotoxic effects of rose essential oil, whole germinated seeds of barley with root meristems were treated with essential oil in concentrations from 250 to 1000 μg/ml. To assess the protective potential of rose oil germinated seeds were conditioning treated with 250 and/or 500 μg/ml for 60 min followed by 4h inter-treatment time and subsequent challenge treatment with 50 μg/ml MNNG (60 min). Third part of germinated seeds was pretreated with 250 and/or 500 μg/ml of rose oil (60 min), followed immediately by 50 μg/ml MNNG (60 min) without any inter-treatment time. For chromosome aberrations evaluation the germinated seeds were treated at 24 °C for 2 hours with 0.025% colchicine in a saturated solution of α-bromonaphthaline after the treatment and recovery times for 18, 21, 24, 27 and 30 hours. The extracted embryos were fixed in a mixture of ethanol and acetic acid (3:1), hydrolyzed in 1N HCl at 60°C for 9 min and stained with Schiff's reagent at room temperature for 1h. The root tips were macerated in a 4% pectinase solution for 12 min and squashed onto slides for scoring of metaphases with chromatid aberrations. For scoring of micronuclei, the root tips were fixed after 30h recovery time without colchicine treatment.

Human lymphocytes in vitro as a test-system. Lymphocyte cultures ($1x10^6$ mol/l) were prepared from venous blood of three healthy nonsmoking/nondrinking donors (men and women) aged between 33 to 50 years according to the standard method of Evans (1984). Each

culture contained 3.5 ml RPMI 1640 medium, heat-inactivated fetal bovine serum (20%), phytohemagglutinin PHA (0.1%) and 40 mg/ml gentamycin (Pharmacia, Bulgaria). The study complies with the Declaration of Helsinki. Voluntary written informed consent was taken from all study participants.

Experimental designs used for cytogenetic analysis. Lymphocyte cultures were treated with rose oil in a range of concentrations from 50 to 500 μg/ml to assess cytotoxic and genotoxic effect of *Rosa alba* L. essential oil. To study its protective potential, non/or low cytotoxic concentrations of rose essential oil - 50 and/or 200 μg/ml were applied as a conditioning treatment (60 min) followed by 4 hours inter-treatment time and a challenge treatment (60 min) with 50 μg/ml MNNG. Another part of the lymphocyte cultures was pretreated with 50 and/or 200 μg/ml of rose oil (60 min), followed immediately by MNNG 50 μg/ml (60 min) without any inter-treatment time. After each treatment the lymphocyte cells were washed with a fresh RPMI medium. Untreated cells were used as a negative control. At the 72nd hour of cultivation to each culture was added 0.02% colchicine, then the cells were hypotonized in 0.56 % KCl; afterwards fixed in a mixture of methanol: glacial acetic acid (3:1, v/v) and stained in 2% Giemsa for assessment of chromosome aberrations. Lymphocytes were directly fixed without colchicine for assessment of micronuclei.

*Cytogenetic analysis***.** Cytotoxicity of Bulgarian *Rosa alba* L. essential oil for both test-systems mentioned above was assessed by mitotic index (MI) using a formula: MI‰=A/1000, where A is a number of dividing cells.

Genotoxic effect was evaluated by chromosome aberrations (CA) and micronuclei (MN) induction. Percentage of metaphases with chromosome aberrations (MwA % ± SD) was calculated. 1000 well spread metaphases (in M1 mitosis) of each treatment variant in both test-systems were assessed. Chromatid breaks (B'), isochromatid breaks (B"), translocations (T), intercalary deletions (D), duplication-deletions (DD) and dicentrics (DC) were determined.

"Aberration hot spots" in the plant chromosomes were determined to obtain information about the DNA segments with higher susceptibility to DNA damage. For analyzing the locus specificity of aberration induction the metaphase chromosomes of *H. vulgare* were subdivided into 48 segments of approximately equal sizes. The segments are numbered with respect to their position in the standard karyotype as described earlier by Künzel and Nicoloff (1979).

Percentage of micronuclei was calculated (MN % ± SD) where 4000 nuclei per experiment and treatment variant were assessed.

Data analysis

The results were calculated statistically by Student's t-test and chi-square method. All experiments were repeated three times. An adapted formula was used for comparison of the upper limit of the confidence interval of the expected and observed chromatid aberrations in individual loci and evaluation of aberration „hot spots" in barley (Rieger *et al.* 1975; Künzel and Nicoloff 1979; Jovtchev *et al.* 2010). For multiple comparisons, a one-way analysis of variance (ANOVA) was employed, followed by Bonferroni`s correction.

RESULTS

Chromatogaphic profile of Rosa alba L. essential oil

The chromatographic composition of the essential oil of Bulgarian *Rosa alba* L. is presented in Table 1. The values were compared with literature data. The main groups are aliphatic hydrocarbons (AH) – 40.92% with high molecular weight (C15 – C27) as heneicosane (12.75%), tricosane (2.69%), eicosane (1.46%), pentacosane (1.0%), heptacosane – 0.86%, (Z)-3-heneicosene-0.64%, etc., followed by oxygenated monoterpenes (OM) – 40.35% as citronellol – 17.69 %, geraniol –16.64 %, trans-citral – 2.12%, linalool – 1.37%, β-citral – 0.68%, etc., sesquiterpenes hydrocarbons (SH) – 5.68% as caryophyllene – 1.52%, α-muurolene – 0.41%, b-cubebene – 0.14%, etc., and oxygenated sesquiterpenes (OS) – 1.72%.

The remaining 11.27% of the constituents were not reported due to their low content, less than 0.05% by mass, and/or not sufficiently reliably identification. After squalene some constituents leaving the column were in fact overlay with the column bleed and were also discarded from the final results.

Superoxide scavenging analysis of Rosa alba L. essential oil

The decrease of absorbance at 560 nm in semples with antioxidant supplements indicates the consumption of superoxide anion radical in the reaction mixture. As can be seen from Figure 1, *Rosa alba* L. essential oil exhibits moderate activity in superoxide scavenging assay in concentration range of 0–500 μg/ml. It is sig-

Table 1. Volatile oil constituents of Bulgarian *Rosa alba* L. essential oil

№	Name	Class	*R. alba* oil, relative percetage	Kovats RI (measured)	Kovats RI (literature)	Reference
1	Linalool	OM	1.37	1092	1099	Babushok *et al.* 2011
2	cis Rose oxide	HM	0.06	1124	1128	Babushok *et al.* 2011
3	a-Terpineol	OM	0.27	1186	1190	Babushok *et al.* 2011
4	Citronellol	OM	17.69	1227	1228	Babushok *et al.* 2011
5	β-Citral	OM	0.68	1244	1249	Jalali-Heravi *et al.* 2006
6	Geraniol	OM	16.64	1256	1255	Babushok *et al.* 2011
7	trans-Citral	OM	2.12	1269	1276	Bertuzzi *et al.* 2013
8	Citronellyl format	OM	0.23	1277	1277	Babushok *et al.* 2011
9	Geranyl formate	OM	0.31	1289	1303	Babushok *et al.* 2011
10	Citronellol acetate	OM	0.12	1346	1352	Babushok *et al.* 2011
11	3,7-dimethyl-2,6-Octadien-1-ol acetate	OM	0.92	1380	1385	Wannes *et al.* 2009
12	Tetradecane	AH	0.05	1400	1400	
13	Caryophyllene	SH	1.51	1419	1420	Babushok *et al.* 2011
14	b-Cubebene	SH	0.14	1430	1434	Facey *et al.* 2005
15	Humulene	SH	0.16	1453	1453	Babushok *et al.* 2011
16	Naphthalene, 1,2,3,4,4a,5,6,8a-oct	SH	0.33	1479	1480	Marongiu *et al.* 2006
17	α-Muurolene	SH	0.41	1504	1498	Babushok *et al.* 2011
18	g-Cadinene	SH	0.19	1528	1523	Babushok *et al.* 2011
19	Salvial-4(14)-en-1-one	SH	0.19	1557	1563	Pavlovic *et al.* 2006
20	Caryophyllene oxide	SH	2.75	1580	1580	Babushok *et al.* 2011
21	3-Octadecine	AH	0.15	1615		
22	Heptadecane	AH	0.54	1700	1700	
23	(2Z,6E) Farnesol	OS	1.72	1722	1722	Babushok *et al.* 2011
24	Z-5-Nonadecene	AH	5.20	1880	1885	Tigrine-Kordiani *et al.* 2006
25	Nonadecane	AH	12.18	1900		
26	3-Nonadecyne	AH	0.57	1911		
27	3-Eicosene, (E)-	AH	0.28	1953		
28	Eicosane	AH	1.46	2000		
29	10-Heneicosene (c,t)	AH	0.34	2070		
30	(Z)-3-Heneicosene	AH	0.64	2085		
31	1-Heneicosene	AH	0.55	2091		
32	Heneicosane	AH	12.75	2100		
33	Docosene	AH	0.41	2200		
34	9-Tricosene, (Z)-	AH	0.74	2290		
35	Tricosane	AH	2.69	2300	2300	
36	1-Pentacosene	AH	0.19	2490		
37	Pentacosane	AH	1.00	2500	2500	
38	Hexacosane	AH	0.09	2600	2600	
39	1-Heptacosene	AH	0.08	2697		
40	Heptacosane	AH	0.86	2700	2700	
41	Octacosane	AH	0.15	2800	2800	

Heterocyclic monoterpenes (HM) – 0.06 %
Oxygenated monoterpenes (OM) – 40.35%
Sesquiterpenes hydrocarbons (SH) – 5.68%
Oxygenated sesquiterpenes (OS) – 1.72%
Aliphatic hydrocarbons (AH) – 40.92%
Total identified - 88.73%

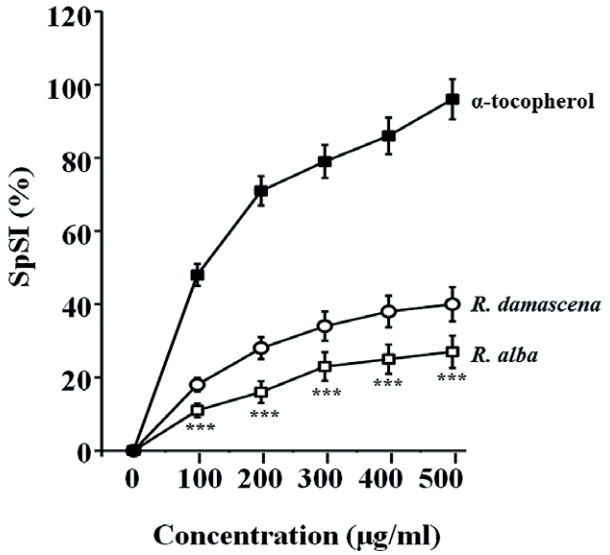


Figure 1. Superoxide scavenging activity of *Rosa alba* L. essential oil. Data were calculated in percentage as spectrophotometric scavenger index (SpSI) – the ratio of the absorption at 560 nm for the sample with oil, and the same absorption for the controls (without oil). The essential oil from Rosa damascena Mill. was used as a control. Data are expressed as mean ± SD of three independent experiments. ***p<0.001 compared with alpha -tocopherol.

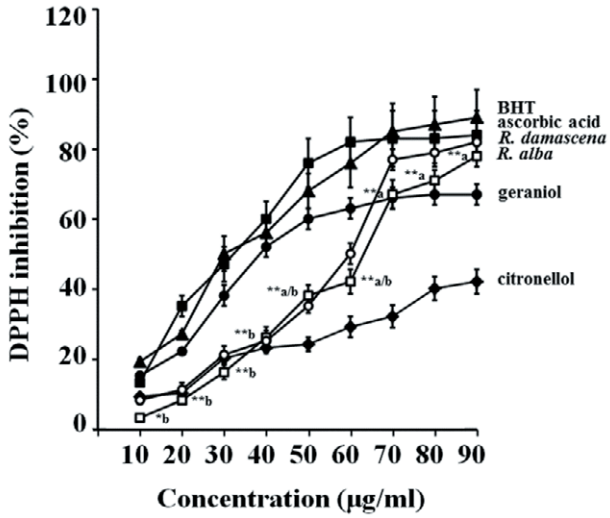


Figure 2. DPPH scavenging activity (%) at various concentrations (µg/ml) of Bulgarian *Rosa alba* L. essential oil and its main ingredients geraniol and citronellol. The essential oil from *Rosa damascena* Mill. was used as a control. Data are expressed as mean ± SD of three independent experiments. ***p<0.001 compared with citronellol (a), compared with BHT and ascorbic acid (b).

nificantly lower than that of alpha tocopherol, but close to that of the *Rosa damascena* Mill. *Our* previous works have shown that essential oils of rose species from different origins in principal does not have high potential as a scavenger of superoxide anion radical (Mileva *et al.* 2014).

DPPH – Radical scavenging assay

Rosa alba L. oil, as well as citronellol and geraniol as main aromatic components of the essential oil were tested in terms of their DPPH – radical scavenging activity. The DPPH assay usually involves a hydrogen atom transfer reaction (Li *et al.* 2009). DPPH radical scavenging test is a sensitive antioxidant assay and depends on substrate polarity. As can be seen from the chromatographic composition of the essential oil, it is rich of components which possess ideal structural chemistry for DPPH radical scavenging activity (Table 1). The presence of multiple hydroxyl functions could be considered as an option for hydrogen donation and/or radical scavenging activity. As can be seen in Figure 2, we found that Bulgarian *Rosa alba* L. essential oil exhibits higher potency in scavenging DPPH radicals than geraniol and citronellol in the applied system, but significantly lower than the benchmark substances (BHT and ascorbic acid). In the concentrations in range of up to 50 µg/ml, *Rosa alba* L. and *Rosa damascena* Mill. rose oils have close activity, but in higher concentracions, over than 50 µg/ml, *Rosa damascena* Mill. oil exhibits about 10% higher activity.

Iron binding capacity of Rosa alba L. essential oil

One of the possible mechanisms of the antioxidant activity of essential oils is the chelation of transition metals. Among the transition metals, iron is known as the most active pro-oxidant, due to its high reactivity. The ferrous form of iron accelerates lipid peroxidation by breaking down hydrogen peroxide and lipid peroxides to reactive free radicals by Fenton's reaction (Li *et al.* 2010). The products of these reactions are able to oxidise cell lipid membranes, modify proteins, as well as to damage DNA. Chelating agents may inactivate metal ions and potentially inhibit the metal-dependent processes (Li *et al.* 2010). Ferrous ion chelating activities of the oil, citronellol and EDTA are shown in Figure 3. The chelating test provided that *Rosa alba* L. essential oil inhibits lipid peroxidation up to 75%. The antioxidant activity obtained in this test is significantly lower than the activity of EDTA but similar to this of citronellol. In the concentration in range of up to 50 µg/ml the two rose oils have close activity, but in higher than 50 µg/ml concentrations *Rosa alba* L. exhibits increasing chelating activity.

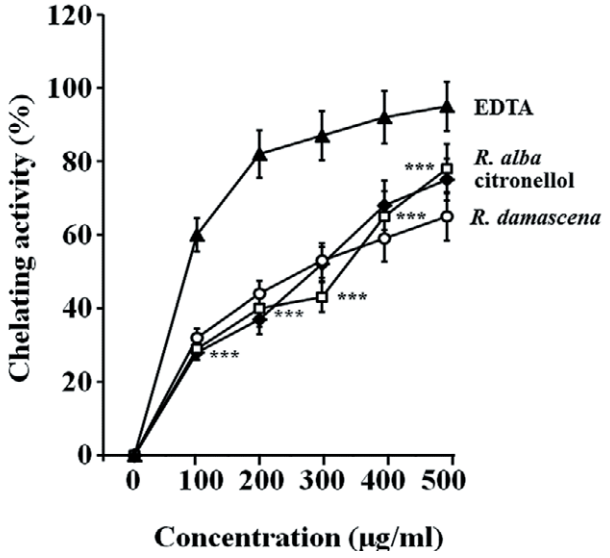


Figure 3. Chelating activity of Bulgarian *Rosa alba* L. essential oil and its main constituent citronellol. The essential oil from *Rosa damascena* Mill. was used as a control. Data are expressed as mean ± SD of three independent experiments. ***p<0.001 compared with EDTA.

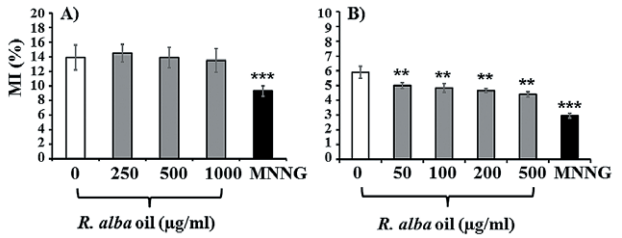


Figure 4. Value of mitotic activity (MI) observed after *Rosa alba* L. essential oil treatment in *Hordeum vulgare* (A) and in human lymphocyte cultures (B). Mitotic activity is calculated as a percent of the control. Data are expressed as mean ± SD of three independent experiments. **p<0.01; ***p<0.001 compared with the negative control.

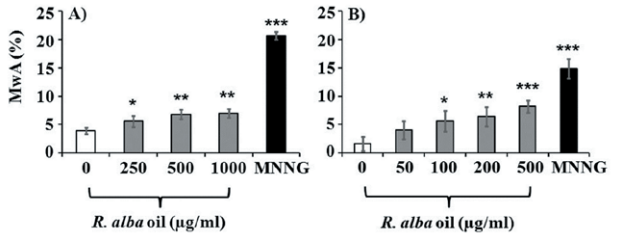


Figure 5. Frequency of chromosome aberrations (MwA) calculated after *Rosa alba* L. essential oil treatment in *Hordeum vulgare* (A) and human lymphocyte cultures (B). Data are expressed as mean ± SD of three independent experiments.*p<0.05; **p<0.01 and ***p<0.001 compared with the negative control.

Cytotoxic and genotoxic effects of Rosa alba L. essential oil

Rosa alba L. essential oil didn't show any cytotoxic effect in barley (Figure 4A). Here, human lymphocytes were found to be more susceptible than barley to rose oil in a concentration range (50-500 µg/ml) used in the present study. The rose oil decreased in a low extent the value of mitotic activity (Figure 4B) compared with the negative control in the lymphocyte cells ($p < 0.01$), whereas after treatment with MNNG (50 µg/ml) a well-expressed cytotoxic effect in both test-systems was observed ($p < 0.001$) (Figure 4A, 4B).

Rosa alba L. essential oil treatment enhanced the induction of chromosome aberrations (MwA) compared to non-treated cells in both test – systems. These results showed its genotoxic effect ($p < 0.05$; $p < 0.01$, $p < 0.001$). The effect is clearly depended on the concentration applied (Figure 5A, 5B). Human lymphocyte cultures were more sensitive to *Rosa alba* L. oil than *Hordeum vulgare*. Genotoxic effect of rose oil was much lower ($p < 0.001$) than the alkylating agent MNNG (50 µg/ml) in both test-systems (Figure 5A, 5B).

Analysis of the chromosome aberrations distribution showed that in barley, rose essential oil induced only isochromatide breaks (data not shown). In human lymphocytes the observed chromosome aberrations were preferably B" 92.0 %, followed by B' 8.0 %. In comparison with the rose oil, MNNG treatment (50 µg/ml) induced more diverse spectrum of chromosome disturbances in both test-systems. In *Hordeum vulgare* were obtained predominantly B"97.0 %, followed by B' 3.0%, whereas in lymphocyte cultures B" were 88.6%, B'- 10.4%, DC-1%, respectively (data not shown) (Figure 6A, 6B).

Treatment with rose oil enhanced the frequency of micronuclei (MN) clearly depending on the test-system and the concentration applied (Figure 6A, 6B; Figure 7A, 7B). No increase of the frequencies of this endpoint were observed in barley meristem cells (Figure 7A). The formation of micronuclei increased ($p < 0.001$) above two-fold (1.1%±0.3 for 50 µg/ml to 1.7%±0.2 for 500 µg/ml) compared to the negative control (0.5%±0.2) in lymphocyte cultures. The genotoxic effect of rose oil assessed as micronuclei is much lower than MNNG in the concentration applied in our study ($p < 0.001$) (Figure 7B).

Anti-cytotoxic and anti-genotoxic potentials of Rosa alba L. essential oil

Anti-cytotoxic potential

Two types of experimental schemes were applied: i) conditioning treatment with non- toxic or low toxic

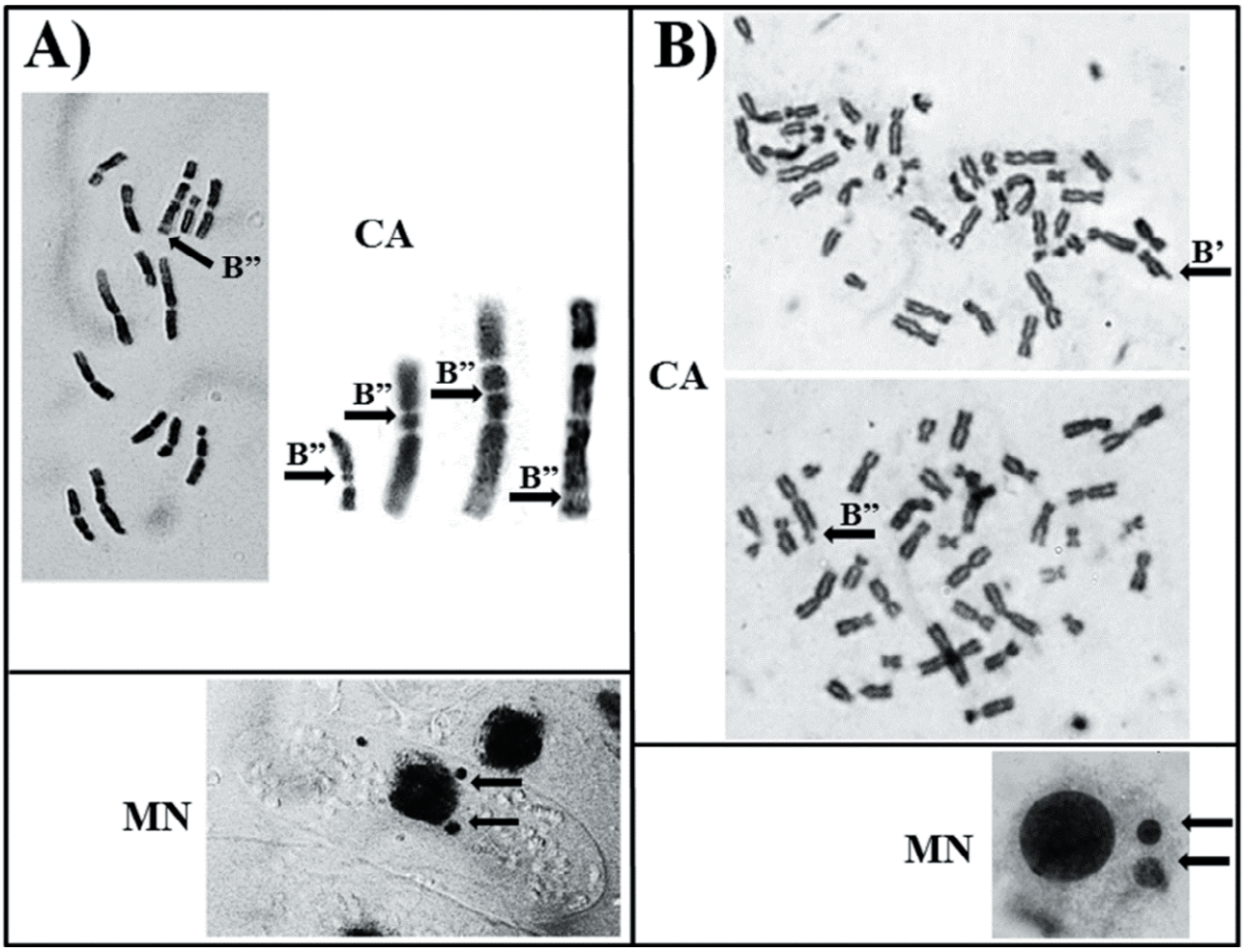


Figure 6. Types of chromosome aberrations (CA) and micronuclei (MN) observed after treatment with *Rosa alba* L. essential oil in *Hordeum vulgare* (A) and in human lymphocyte cultures (B).

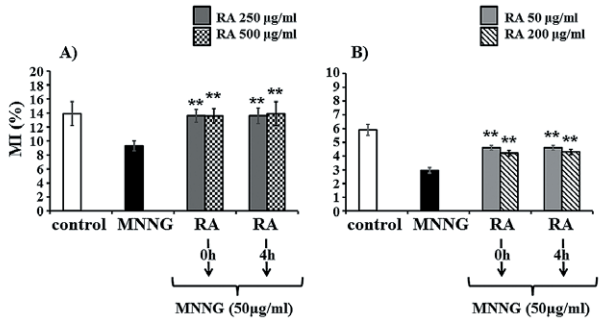


Figure 7. Induction of MN observed after *Rosa alba* L. essential oil treatment in *Hordeum vulgare* (A) and in human lymphocyte cultures (B). Data are expressed as mean ± SD of three independent experiments. **p<0.01 and ***p<0.001 compared with the negative control.

concentrations of rose essential oil followed by challenge treatment with alkylating agent MNNG (50 µg/ml) and 4 hours inter-treatment time, ii) treatment without any inter-treatment time between treatments (Figure 8A, 8B).

Mitotic activity (MI) observed after treatment showed clear dependence on the experimental design and test-systems (Figure 8A, 8B). The value of mitotic index was significantly increased ($p < 0.01$) after treatment applying both schemes of experimental design, compared to those obtained after MNNG (50 µg/ml) treatment alone in both test-systems (Figure 8A, 8B). A lack of any difference was obtained between the values of mitotic activity after rose essential oil conditioning treatment (250, 500 µg/ml for barley and 50, 200 µg/ml for human lymphocytes, respectively) followed by challenge treatment with MNNG (50 µg/ml) with 4 hours

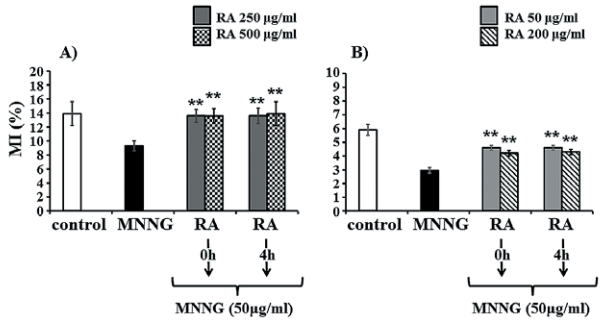


Figure 8. Anti-cytotoxic potential of rose essential oil assessed by mitotic index (MI) applying experimental schemes with: - *Rosa alba* L. essential oil conditioning treatment prior to MNNG challenge (50 µg/ml) with 4 hours inter-treatment time and, - without any inter-treatment time in *Hordeum vulgare* (A) and in human lymphocyte cultures (B). Mitotic activity is calculated as a percent of the control. Data are expressed as mean ± SD of three independent experiments. **p<0.01 compared with MNNG.

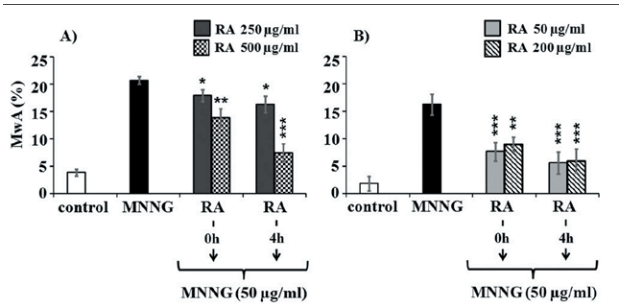


Figure 9. Anti-genotoxic potential of rose essential oil assessed by induction of chromosome aberrations (MwA) applying experimental schemes with: - *Rosa alba* L. essential oil conditioning treatment prior to MNNG challenge (50 µg/ml) with 4 hours inter-treatment time and, - without any inter-treatment time in *Hordeum vulgare* (A) and in human lymphocyte cultures (B). Data are expressed as mean ± SD of three independent experiments. *p<0.05, **p<0.01 and ***p<0.001 compared with MNNG.

inter-treatment time and treatment without any inter-treatment time.

Anti-genotoxic potential

Significantly ($p < 0.05$ till $p < 0.001$) lower frequency of chromosome aberrations was observed in *Hordeum vulgare* after conditioning treatment with rose essential oil (250, 500 µg/ml) prior to MNNG challenge (50 µg/ml) with 4 hours inter-treatment time, compared to that induced after treatment with alkylating agent only (Figure 9A). The reduction of MNNG induced chromosome aberrations was nearly three times lower in samples after rose oil 500 µg/ml conditioning (7.5%±1.6) compared to MNNG single treatment (20.7%±2.0).

Similar anti-genotoxic effect ($p < 0.001$) was observed in human lymphocytes after applying the same experimental scheme of treatment – conditioning with rose essential oil (50, 200 µg/ml) followed by challenge with MNNG (50 µg/ml) with 4 hour inter-treatment time (Figure 9B). Chromosome injuries were decreased approximately three times in samples conditioned with rose oil 50 µg/ml (5.6%±1.7) and with 200 µg/ml (6.0%±1.3) compared to MNNG treatment alone (16.3%±1.9). Lower structural chromosome disturbances were also calculated after treatment with rose essential oil and alkylating agent without any inter-treatment time (Figure 9A). The frequencies of chromosome aberrations were decreased between 2.2-times for samples conditioned with 50 µg/ml rose oil to 1.8–times for samples conditioned with 200 µg/ml. Reduction of the frequencies of chromosome aberrations was observed in barley cells but to a lower extent ($p < 0.01$) also after treatment following the scheme without any inter-treatment time compared to those after MNNG treatment alone (Figure 9A).

The spectrum of chromosome disturbances induced after applying experimental schemes for assessing anti-genotoxic potential of rose essential oil (250, 500 µg/ml) in *H. vulgare* showed predominantly B" 93%, followed by T 4% and B' 3% in samples with 4 hours inter-treatment time, and B" 93%, T-5%, D-1%, B'-1% in samples without any inter-treatment time (data not shown). In human lymphocytes conditioning treated with rose oil (50, 200 µg/ml) followed by MNNG and inter-treatment time of 4 hours were obtained B" 84.4%, B'-11.8%, T-1.9%, RC-1% respectively, whereas in samples treated with rose oil and MNNG without any inter-treatment tine were observed mainly B" 85.5%, B' 12.1% and T 2.4% (data not shown).

By calculating the frequency of micronuclei as another endpoint for genotoxicity, it was observed that rose essential oil showed similar anti-genotoxic effect ($p < 0.01$, $p < 0.001$) in both test-systems (Figure 10A, 10B). The effect was obtained applying both schemes of experimental design. In *Hordeum vulgare* conditioning treatment with rose essential oil decreased MN approximately two-times (0.97%±0.12 for 250 µg/ml and 1.02%±0.10 for 500 µg/ml) compared to that induced after treatment with the alkylating agent (1.95%±0.18). Similarly, lower MN frequency was obtained after treatment of barley cells without any inter-treatment time (Figure 10A). Frequency of micronuclei was decreased roughly 2 times – 1.0%±0.11 for 250 µg/ml and 1.04%±0.14 for 500 µg/ml. In human lymphocytes conditioning treatment with rose

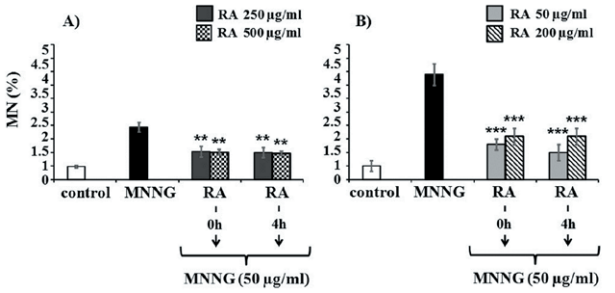


Figure 10. Anti-genotoxic potential of rose essential oil assessed by induction of micronuclei (MN) applying experimental schemes with: - *Rosa alba* L. essential oil conditioning treatment prior to MNNG challenge (50 µg/ml) with 4 hours inter-treatment time and, - without any inter-treatment time in *Hordeum vulgare* (A) and in human lymphocyte cultures (B). Data are expressed as mean ± SD of three independent experiments. **p<0.01; ***p<0.001 compared with MNNG.

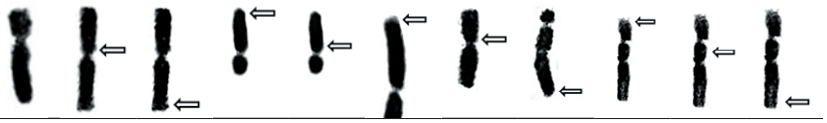
oil decreased MN approximately four-times 1.0%±0.2 for 50 µg/ml and 1.6%±0.3 for 200 µg/ml compared with those induced by MNNG alone (3.9%±0.4). The frequency of micronuclei was from 3-fold (for 50µg/ml) to 2.4-fold (200 µg/ml) lower than that of MNNG after treatment without any inter-treatment time (Figure 10B).

"Aberration Hot Spots" in Barley. "Aberration hot spots" are a good expression tool to investigate genotoxic activity as well as anti-genotoxic potential of *Rosa alba* L. essential oil. They give additional information about the effect of the essential oil on *Hordeum vulgare* root meristem cells.

The potential of MNNG to induce "aberration hot spots" in plant chromosomes was analyzed in the reconstructed barley karyotype MK14/2034. Seven out of the 48 inspected segments showed significant deviation from a random distribution of isochromatid breaks. Aberration clustering of isochromatid breaks (Table 2) was found in segment 10 and segment 14 of chromosome 2 (4.5% and 5.8%, respectively), in segment 17 of chromosome 3^4 (6.4%), in segment 21 of chromosome 4^3 (4.2%), in segment 30 chromosome 5 (9.1%), and in segments 44 and 48 of chromosome 7^1 (7.3%, resp. 6.7%).

All of these segments are located directly adjacent to the centromeres in the heterochromatin rich regions and are not connected with the regions of chromosome reconstruction. After *R. alba* L. essential oil treatment alone concentration dependent aberration hot spots (2 or 3, resp.) were observed (see Table 2), namely: after treatment with *R. alba* L. oil 250 µg/ml – segment 30 of chromosome 5 (8.2%) and segments 41 of chromosome 6 (14.2%); after treatment with *R. alba* oil 500 µg/ml – segment 14 of chromosome 2 (6.2%), segment 21 of chromosome 4^3 (6.2%) and segment 30 of chromosome 5 (10.0%)and after treatment with *R. alba* L. essential oil 1000 µg/ml – segment 17 of chromosome 3^4 (7.2%),

Table 2. Observed aberration "hot spots" in chromosomes of barley root tip meristem cells of the reconstructed karyotype MK14/2034 after different treatment procedures

Treatment variants		Non-Spot segments	Hot spot segments									
												
			Chr.1^7	Chr.2 seg. 10	Chr.2 seg. 14	Chr.3^4 seg. 15	Chr.3^4 seg. 17	Chr.4^3 seg. 21	Chr.5 seg. 30	Chr.6 seg. 41	Chr.7^1 seg. 1	Chr.7^1 seg. 44	Chr.7^1 seg. 48
1.	control	100%											
2.	MNNG (50 µg/ml)	56.0%		4.5%	5.8%		6.4%	4.2%	9.1%			7.3%	6.7%
3.	*Rosa alba* oil (250 µg/ml)	77.6%							8.2%	14.2%			
4.	*Rosa alba* oil (500 µg/ml)	77.6%			6.2%			6.2%	10.0%				
5.	*Rosa alba* oil (1000 µg/ml)	72.2%					7.2%		10.3%			10.3%	
6.	*Rosa alba* oil (250 µg/ml) → MNNG (50 µg/ml)	60.3%				5.6%		6.3%	6.3%		6.9%		14.6%
7.	*Rosa alba* oil (250 µg/ml) → 4h IT → MNNG (50 µg/ml)	60.6%			10.5%	11.4%				7.9%			9.6%
8.	*Rosa alba* oil (500 µg/ml) → MNNG (50 µg/ml)	73.0%		8.5%	8.5%							10.0%	
9.	*Rosa alba* oil (500 µg/ml) → 4h IT → MNNG (50 µg/ml)	81.5%							7.6%			10.9%	

segment 30 of chromosome 5 (10.3%) and segment 41 of chromosome 6 (10.3%).

Conditioning treatment with *R. alba* L. essential oil in concentrations of 250 μg/ml and 500 μg/ml, without any inter-treatment time prior to MNNG challenge decreased the aberration "hot spots" to five or three out of the 48 inspected segments (Table 2): *R. alba* L. essential oil 250 μg/ml – segment 15 chromosome 3^4 (5.6%), segment 21 of chromosome 4^3 (6.3%), segment 30 of chromosome 5 (6.3%), segments 1 and 48 of chromosome 7^1 (6.9% and 14.6%, resp.); *R. alba* L. oil 500 μg/ml – segments 10 and 14 chromosome 2 (both 8.5%) and segment 48 of chromosome 7^1 (10.0%). After inter-treatment time of 4 hours between conditioning and challenge treatment only 4 aberrations "hot spots" for conditioning with *R. alba* L. oil 250 μg/ml showed a significant deviation from a random distribution of isochromatid breaks – segment 14 of chromosome 2 (10.5%), segment 15 of chromosome 3^4 (11.4%), segment 41 of chromosome 6 (7.9%) and segment 48 of chromosome 71 (9.6%). Two aberrations "hot spots" were found after conditioning with *R. alba* L. essential oil 500 μg/ml, namely segment 30 of chromosome 5 (7.7%) and segment 44 of chromosome 7^1 (10.9%) (Table 2).

"Aberration hot spots" were found in all treated variants in barley chromosomes – karyotype MK 14/2034, with exception of chromosome 1^7 (Table 2).

In summary, applying both experimental schemes for assessing anti-genotoxic effect of *R. alba* L. essential oil the frequency of aberration "hot spots" was statistically significant decreased compared to MNNG 50 μg/ml (7 aberration "hot spots") (see Table 2). After consecutive treatment *R. alba* L. oil 500 μg/ml – 4 h inter-treatment time – MNNG the best result was achieved – reduction of aberration "hot spots" to 2 (Table 2).

DISCUSSION

In the current study valuable information was obtained about cytoprotective/genoprotective and antioxidant potentials of *Rosa alba* L. essential oil. The use of two types of test-systems – barley and human lymphocytes *in vitro,* which are widely applied in the genotoxic studies makes evaluation more representative.

Our results showed that rose essential oil possessed good expressed DPPH radical scavenging activity; the effect increased with the increasing of the concentration. It confirmed the data of Hatano *et al.* (1989) who proposed that rose's extract radical scavenging ability is due to the polar-bounded hydrogen. The compounds, which are able to donate hydrogen, are able to break the chain reaction of lipid peroxidation at the first initiation step, and/or to produce redox-silent compounds. Gordon (1990) reported that the chelating agents are effective as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of the metal ion. The data shown in Figure 3 reveal that the Bulgarian *Rosa alba* L. oil demonstrates an effective capacity as iron binding agent, depending on the concentration applied, so its behavior as liposomal membrane peroxidation protector could be due to its iron binding capacity (Mileva *et al.* 2014). Moreover, in concentrations higher than 400μg/ml *Rosa alba* L. oil has a higher activity than *Rosa damascena* Mill.

In the present study Bulgarian *Rosa alba* L. essential oil didn't show significant cytotoxic effect in barley but it has relatively low cytotoxicity in human lymphocytes *in vitro* depending on the concentration. Sinha *et al.* (2014) demonstrate that essential oils may be safe at low concentrations, but show toxicity to humans at high concentrations represented as lethal doses. As typical lipophiles, essential oils can pass through the cell membrane and cytoplasmic membrane. They disrupt the structure of the different layers of polysaccharides, fatty acids and phospholipids and permeabilize through them (Bakkali *et al.* 2008). Their mode of action affects several targets at the same time. The cytotoxic activity of some essential oils for example of *Ocotea quixos* and others is mostly due to the presence of phenols, aldehydes and alcohols (Bruni *et al.* 2003; Sacchetti *et al.* 2005). In our study aliphatic hydrocarbons (AH) are the major compounds of *Rosa alba* L. essential oil. Here heneicosane (12.75%) and tricosane (2.69%) are representatives of the main components of this group (Table 1). Similar oil composition but in higher concentrations for some ingredients was detected for *Rosa damascena* Mill. by Kovacheva *et al.* (2010; 2011) and Mileva *et al.* (2014). The essential oils of plants such as *Ceratonia siliqua* (Hsouna *et al.* 2011), *Ailanthus altissima* (Albouchi *et al.* 2013) and *Viscum album* leaves (Cebovic *et al.* 2008), contain the same compounds in similar concentrations as in the white rose oil. They showed obvious cytotoxic effects, high antioxidant and phytotoxic activities. Shokrzadeh *et al.* (2017) reported a sensitivity of cancer cell line (A549) to high concentrations of rose oil obtained from *Rosa damascene* Mill. from Kashan. Probably these substances play a role in the cytotoxicicity of rose essential oil that we obtained for human lymphocytes. The higher resistance of *Hordeum vulgare* cells compared to the human lymphocytes is probably due to their different cell permeability and a cell wall existence compared to lymphocyte cells.

The current investigation detects genotoxic activity of Bulgarian *Rosa alba* L. essential oil depending on

the concentrations in both test-systems applied. Shokrzadeh *et al.* (2017) also reported that at concentrations of 50-200 μg/ml *Rosa damascena* Mill. oil significantly increased the frequency of micronuclei in human lymphocytes. According to Bakkali *et al.* (2008) in the case of cytotoxicity and genotoxicity, essential oils can damage the cellular and organelle membranes and can act as pro-oxidants on proteins and DNA *via* production of reactive oxygen species (ROS).

As it is known numerous plant extracts or phytochemicals have dual aspects, showing both genotoxicity and anti-genotoxicity against mutagens and carcinogens *in vivo* and *in vitro* test-systems (Kopaskova *et al.* 2012; Reddy *et al.* 2014; Gateva *et al.* 2015). Here we made an attempt to study the defense potential of Bulgarian *Rosa alba* L. essential oil and to determine the experimental conditions under which it can occur against alkylating agent MNNG. This is a typical mutagenic agent, which damages DNA. As a result it induced intra-strand, inter-strand crosslinks and double-strand breaks as well as base methylations, respectively (Kinzella and Radman 1980; Black *et al.* 1989). Extracts and essential oils of various plants were used to decrease cytotoxicity and genotoxicity induced by numerous genotoxins including alkylating agents (Vicuña *et al.* 2010; Mezzoug *et al.* 2007; Leffa *et al.* 2012; Madrigal-Santillán *et al.* 2013; Agabeyli 2012; Kuzilet *et al.* 2013; Matić *et al.* 2015).

The current results clearly show anti-genotoxic potential of Bulgarian *Rosa alba* L. essential oil manifested by decreasing of the frequencies of chromosome aberrations and micronuclei after conditioning treatment with rose oil before MNNG challenge and 4 hours inter-treatment time in both experimental test-systems used by us. This is in agreement with our study (Gateva *et al.* 2019) where the preventive effect of acyclic monoterpenoid geraniol (which is one of the major rose essential oil compounds) was obtained against MNNG in human lymphocytes *in vitro* and *Hordeum vulgare*. Geraniol was found to be effective in limiting the genotoxic effect of MNNG applied as conditioning treatment (4h inter-treatment time) prior challenge with MNNG compared to the samples treated with MNNG alone. Our current study also showed an increased resistance to the damaging effect of MNNG both in barley and in human lymphocytes after treatment with rose essential oil and MNNG without any inter-treatment time. Hence the defence potential of Bulgarian *Rosa alba* L. essential oil is manifested regardless of the experimental conditions. The results about anti-cytotoxic and anti-genotoxic effect of the rose oil corresponded with those for antioxidant activity of rose essential oil obtained by DPPH test and iron chelating analysis. As a rule, the anti-cytotoxic, anti-genotoxic, and antioxidant properties of the plant extracts cannot be attributed of activities to single constituents. Their biological activity could be explained with the combination of effects to one another. As can be seen on chromatographic profil of *Rosa alba* L. oil (Table 1), geraniol and citronellol are in major amounts in oil, so they could have an over additive participation in total antioxidant and iron chelating properties. Ruberto and Baratta (1999) demonstrated that most radical scavenging activities of essential oils are mainly due to the cumulative effect of ingredients nerol, citronellol and geraniol, within whose structure polar bounded hydrogen has been observed. Undoubtedly, DPPH radicals have little relevance as presence in biological systems, but the results are indicative of the capacity of the Bulgarian white rose oil to scavenge free radicals which relate to hydrogen atom or electron donation ability.

There are data indicating that not only geraniol and citronellol, but citral (which belongs to the bioactive compounds of the Bulgarian *Rosa alba* L. essential oil) also possess antioxidant activity (Raut and Karuppayil 2014). Madankumar *et al.* (2013) showed that geraniol has a potent antioxidant effect by scavenging oxygen-free radicals and increasing the level of total glutathione content (GSH) in murine skin. It could modulate the activity of enzymatic and non-enzymatic antioxidants to exert its chemopreventive activity against 4-Nitroquinoline 1-oxide induced oral cancer in rats. Manoharan and Selvan (2012) proposed that geraniol inhibits abnormal cell proliferation occurring in skin carcinogenesis by modulating the activities of Phase II detoxification agents and through free radical scavenging potential. Geraniol and camphene were found to significantly decrease lipid peroxidation, inhibit Nitric oxide release (83.84% and 64.61%) and ROS generation in the pre-treated cells as compared to stressed cells (Tiwari and Kakkar 2009). Kashani *et al.* (2011) described a significant correlation between the phenolic content and DPPH scavenging capacity of white rose extracts. Our results indicate good iron chelating and DPPH radical-scavenging activities, medium superoxide scavenging ability of Bulgarian *R. alba* L. essential oil, which is probably due to its chemical composition. Some authors reported that **α**-terpineol (which is one of the white rose essential oil compounds obtained by us) has remarkable ferrous ions chelating agent and possees antimutagenic activity against 2-aminoanthracene in *S. typhimurium* TA100 (Di Sotto *et al.* 2013).

According to Bakkali *et al.* (2008) the mechanism of the decrease of mutagenicity did not depend only of the type of essential oil but on the type of mutagen, thus on the type of lesions and consequently on the DNA repair

or lesion avoidance system involved. The protective effect obtained by us for Bulgarian *Rosa alba* L. essential oil against alkylating agent MNNG suggests an activation of repair pathways in addition to antioxidant and scavenging activity of rose essential oil. It is well known that N^7-alkylG is responsible for about 90% of the total frequency of alkylation events among the alkylation damages induced by alkylating agents. Quantity of O^6-alkylG (DNA adduct formed by the alkylating agent) is less, but if not repaired could led to DNA damage such as cross-linking, strand breaks and modification of bases (Kondo *et al.* 2009). However N^7-alkylG is considered to be as mutagenic as O^6-alkylG because it is efficiently repaired by base excision repair (BER) pathway (Drablos *et al.* 2004). O^6-methylG lesions are repaired by direct damage reversal repair carried out by the enzyme O^6-methylguanine-DNA methyltransferase (MGMT) also referred to as alkylguanine transferase (AGT). MGMT efficiently repairs O^6-methylG before replication, through direct transfer of the adducted methyl group from the oxygen in the guanine to a cysteine residue in the catalytic site of MGMT (Ramos *et al.* 2011). Niture *et al.* (2006; 2007) showed that both the ethanolic and aqueous extracts and compounds of some medical plants increased MGMT expression and its activity in lymphocytes and cancer cell lines. To understand the real mechanism of protective potential of rose essential oil when applied in combination with alkylating agents more studies are needed.

CONCLUSION

Bulgarian *Rosa alba* L. essential oil has low cytotoxicity in barley and human lymphocytes *in vitro* in a dose-dependent manner, as well a good cytoprotective/genoprotective effect against DNA damaging agent MNNG when applied both with 4 hours between treatments and without any inter-treatment time. Anti-genotoxic potential of rose essential oil was manifested by the decrease of the frequency of chromosome aberrations and the micronuclei in both test-systems. Something more, white rose's oil demonstrated well-pronounced anti-oxidant potential and very good metal chelating activity. The results show that rose oil contained protective compounds that can decrease DNA damage. Data suggest a promising ethnopharmacological potential of Bulgarian white rose essential oil. It could serve in medical cosmet as a prophylactic agent, and as an adjuvant in cancer prevention and therapy.

GEOLOCATION

Fresh flowers of *Rosa alba* L., from the experimental field of the Institute of Rose and Essential Oil Plants (IREOP), in Kazanlak, Bulgaria were used. All studies were conducted in the laboratories of the Republic of Bulgaria.

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Display of *Sukkula* distributions on Barley Roots via *in situ* hybridization

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Abstract. Retrotransposon are an abundant and ancient parts of the plant genomes that especially LTR retrotransposons influence the genome size and evolution. *Sukkula* is a non-autonomous and active, relatively high copy-number retroelement. In this study, we performed fluorescence *in situ* hybridization (FISH) to observe the distributions of *Sukkula* elements (*LTRs* and *internal-domain*) by using labelled-PCR products. The localizations of *Sukkula* elements (*LTRs* and *internal-domain*) were observed under confocal microscope on *Hordeum vulgare* L. cv. Hasat root preparations. Our results revealed that *Sukkula* elements is still active and spread through the whole barley chromosomes. Additionally, the re-sequencing analysis of *Sukkula* LTRs demonstrated that LTRs sequences had ~65 bp gain. These analyses represent a valuable resource to reveal genome organization of barley and large sized plants.

Keywords: fluorescence *in situ* hybridization, retrotransposon, *Sukkula*, Barley, *LTRs*, *internal-domain*.

INTRODUCTION

Beginning with the pioneering work of Barbara McClinton, transposable elements (TEs) have become to take part a central position in the plant genome studies. TEs consist of DNA fractions capable of chromosomal movement, either *via* replicative or conservative (cut-and-paste) mechanisms (Doolittle and Sapienza 1980; Orgel and Crick 1980; Finnegan 1989). Eukaryotic TEs contain two main classes; Class I elements and Class II elements. Class I elements are also known as retrotransposons move through using an RNA intermediate, while Class II elements move through the genome using a DNA intermediate (Finnegan 1989).

In plants, the vast majority of repetitive DNA in the nuclear genomes is derived from the proliferation of mostly Class I elements called as retrotransposons (SanMiguel *et al.* 1996; Vicient *et al.* 1999; Hawkins *et al.* 2006; Neumann *et al.* 2006; Vitte and Bennetzen 2006) which are subdivided as two major subclasses; Long terminal repeat (LTR) retrotransposons and non-LTR retrotransposons. LTR retrotransposons, which typically comprise GAG and POL protein coding ORFs encoding several enzymes (reverse transcriptase

– RT; protease – PR; RNaseH – RH; integrase – INT) responsible for reverse transcription and integration of daughter sequences into new chromosomal locations, constitute the largest fraction of the TEs (Eickbush and Malik 2002; Havecker *et al.* 2004; Hawkins *et al.* 2006; Neumann *et al.* 2006; Vitte and Bennetzen 2006). Moreover, LTR retrotransposons are found in plants are subdivided in two main superfamilies, *gypsy*-like and *copia*-like (also known as *Metaviridae* and *Pseudoviridae*, respectively), which include the same protein coding domains. However, these domains are rearranged in different order in both LTR retrotransposon types (Eickbush and Malik 2002; Havecker *et al.* 2004).

Sukkula elements were first identified in barley genome at the barley Mlo locus and to an insertion sequence present in the 3' LTR of one BARE1element (Manninen and Schulman 1993). Shirasu *et al.* (2000) later determined two ~5 kb sequence similar this insertion in a 66 kb stretch of barley genome that were also found to be flanked by 5 kb direct repeats. Therefore, these sequences were named as *Sukkula* elements means "shuttle" in Finnish. However, *Sukkula* LTR copies are found to be *gypsy*-like retrotransposons, but non-autonomous elements belonging to a novel group of retroelements, *l*arge *r*etrotransposon *d*erivatives or LARDs (Kemekawa *et al.* 1999; Kalendar *et al.* 2004). Moreover, *Sukkula* elements consist of reverse transcriptase in appx. 3.5 kb central domain which is found to be conserved as in primary sequence and secondary structure, including no open reading frames (ORFs) encodes typical retroelement proteins. According to these features of *Sukkula* elements, they are TRIMs (Terminal-repeat Retrotranposons in Miniature) in their lack of a protein-coding domain (Kalendar *et al.* 2004). Active retrotransposons are important for genome diversification in plants, because of their transposition and accumulation potentials in the genome, thus it can change the overall genome structure (Wessler *et al.* 1995; Vicient *et al.* 1999; Schulman and Kalendar 2005).

Fluorescence *in situ* hybridization (FISH) using target-specific DNA probes have become important tool in modern biology and cell research (Hausmann and Cremer 2003). In plants, introducing FISH probes is more difficult, because of the cell wall and the cytoplasm of the plants that they hinder chromosome spreading and low metaphase indices (Salvo-Garrido *et al.* 2001). By using FISH technique, the distribution of retrotransposon families has been reported in various plants such as *Hordeum vulgare, Allium cepa, Aegilops speltoides, Brachypodium distachyon* and *Glycine max* (Vicient *et al.* 1999; Lin *et al.* 2005; Kiseleva *et al.* 2014; Shams and Raskina 2018; Li *et al.* 2018). *BARE1* distributions on barley chromosomes have been demonstrated by using BAC clones a probe *via* FISH (Vicient *et al.* 1999). In barley, FISH technique was also used to reveal gene organization and to integrate the genetic linkage map with a physical map (Stephens *et al.* 2004).

The aim of this study was to present the distributions of *Sukkula* elements (*LTRs* and *internal-domain*) in *Hordeum vulgare* L. cv. Hasat chromosomes using labelled-PCR products via FISH. *Sukkula* localization patterns were observed under confocal microscope on barley root preparations. We also performed sequencing studies and the sequence analysis of *Sukkula* elements (*LTRs* and *internal-domain*) to elucidate *Sukkula* sequence alterations in barley. Our results indicate that *Sukkula* elements (*LTRs* and *internal-domain*) are still active and under genome evolution.

MATERIALS AND METHODS

Plant materials

Barley (*Hordeum vulgare* L.) cv. Hasat was provided from Directorate of Trakya Agricultural Research Institute. The seeds were grown at growth chamber for germination period under controlled conditions (16 h light/8 h dark, 25°C ± 2°C) and relative humidity was kept at 60–75%. The plants were harvested after 72 hours, directly treated with liquid nitrogen and then stored at –80°C until DNA extraction.

gDNA Isolation

gDNA were isolated from 200 mg of the samples by using the cetyltrimethylammonium bromide (CTAB) precipitation method was modified as previously described in Mafra *et al.* (2008). Specifically, 200 mg homogenized sample was incubated with 1 ml Edward's buffer (0.5% (w/v) SDS, 250 mM NaCl, 25 mM EDTA, 200 mM Tris pH 8.0) at 95ºC for 5 min (Cold Spring Harbor Laboratory 2005). They were then spun down at relative centrifugal force of 16,000 g in a microcentrifuge for 15 min, and the supernatant was isolated twice with chloroform. Then, the aqueous phase was incubated with 2 volumes of CTAB precipitation solution, after which the CTAB protocol was followed as previously described (Mafra *et al.* 2008). DNA yield and purity were measured by UV spectrophotometry at 230, 260 and 280 nm using a NanoDrop 2000c instrument (Thermo Scientific USA). DNA integrity was evaluated by agarose gel electrophoresis, samples were separated on 1% agarose gels containing Ethidium bromide nucleic acid stain in 1X TAE buffer.

Chromosome preparation for FISH analysis

Grains were placed randomly in petri dishes containing filter paper soaked in only water to germinate in an incubator at 18-25°C in the dark for 3 days. Then, root tips of barley cv. Hasat were harvested, then directly fixed in Carnoy fixative (3:1 ethanol:acetic acid solution) without any chemical pre-treatment, stored roots at 4°C. Chromosome preparations and FISH analysis were performed according to Jenkins and Hasterok (2001, 2007) with modifications. The slides were checked under the light microscope (Olympus U-TVO.5XC-3) and kept in a freezer at -20 °C.

Development of probes and labelling

The FISH probes used in this study were generated from two set of data which is the *Sukkula* (*internal-domain*) gene and *LTR* sequences. To investigate the distribution of *Sukkula*, we amplified *internal-domain* and *LTR* sequences of *Sukkula* using designed specific primer sets Table 1. The probes for *internal-domain* and *LTR* sequences designated by using IDT's PrimerQuest© Tool (2012). GC% and Tm values of probes were around 50 and between 50°C and 55°C, respectively. The sequences of *Sukkula LTR* and *internal-domain* were obtained from barley (AY054376 for *LTR* and *intern-domain*).

Probe synthesis was carried out individually by using *Sukkula LTR* and *internal-domain* primers. The reactions were carried out in total volume of 50 µl including 18.25 µl nuclease-free water, 25 µl of Hot-Start PCR Master Mix (Bio-Rad), 1.5 µl of each primer (10 µM/µl), 1.75 µl of tetramethylrhodamine-dUTP (TRITC) (1 mM), and 2 µl template DNA (40 ng/µl). PCR conditions were as follows: 94°C for 5 min followed by 40 cycles of 94°C for 25 s, annealing 50°C for 25 s and 72°C for 30 s. The reaction was completed by a final extension step at 72°C for 5 min.

Table 1. Primers used in this study.

No	Primer Name	Sequence (5'→3')
1	*Sukkula LTR* F	CCCTCCTTCCCTCTTCTCTAAT
2	*Sukkula LTR* R	CCATACTCTGAACCTGATCCTAAAC
3	*Sukkula LTR sequencing* F	AACCAGTCAACCAGCATAGG
4	*Sukkula LTR sequencing* R	GGAGAGGGAGAGATAAGAGGAA
5	*Sukkula internal-domain* F	CCTTGCACTTGATGGCTACT
6	*Sukkula internal-domain* R	CGGATGAGACACGGAAGAAA

Fluorescence in situ hybridization (FISH) analysis

The FISH analysis procedure was performed based on Jenkins and Hasterok protocol (2001, 2007) with modifications. Chromosome spreads were scanned under ×40 objective light microscopes to define the number and quality of well-spread metaphase plates, and they were treated with 100 µg/ml of RNase at 37°C for 1 h. The hybridization mixture consist of 20 µl of deionised formamide (50%), 8 µl of dextran sulphate (10%), 4 µl of 20X SSC (2X SSC), 2 µl of 10% SDS (0.5%), 10 µl of probe (75-200ng/slide), 1 µl of blocking DNA (sonicated salmon sperm DNA) (25-100X probe) and added sterile dH_2O to bring final volume 40 µl. Final concentrations were indicated in parenthesis. The mixture was denatured at 85°C for 10 min and kept on ice for 10 min. A 38 µl aliquot of the hybridization mixture was applied onto each slide, covered with a coverslip, and sealed with paper bond. Both chromosomal DNA and probe DNA on the slides were denatured together in a thermal cycler at 70°C for 6 min and hybridized with each other at 37°C overnight in a humid dark box. Afterwards, hybridization the chromosome spreads were washed three times in 2X SSC: once 2X SSC to float coverslips off; once in 15% formamide/0.1X SSC, and again once in 15% formamide/0.1X SSC, each for 10 min at 42°C. Then, slides were washed in 2X SSC for 3 min at 42°. This step was repeated twice with fresh 2X SSC at 42°. Ultimately, slides were washed three times in 2X SSC for 3 min at RT. After, slides were dehydrated in alcohol series (70, 90 and 100%), each for 1 min at RT and waited in the dark for 15-20 min. Vectashield-DAPI mounting-staining medium (7-10 µL) was dropped onto the chromosome spreads, which were then stored at 4°C until used.

Image acquisition

For imaging the slides, the following wavelengths were utilized for fluorescence detection: 551-575 nm for probes labelled with TRITC and 420-480 nm for DAPI in Leica DM5500 confocal microscope. The different fluorescent images were acquired separately. Afterwards, they were merged into single composite images. The signal images were analysed by Adobe Photoshop CC 2014.

Sequence analysis

For sequence analysis of *Sukkula LTRs*, we performed PCR reaction. The PCR products were re-sequenced. The sequence homology search was conducted in barley genome by using BLASTN in the Ensembl website (http://plants.ensembl.org/barley). However, the re-sequencing results of *Sukkula LTRs* were compared the original *Sukkula LTR* sequences using Clustalomega (Altschul *et al.* 1990).

RESULTS AND DISCUSSION

Total copy number of TEs in plant genomes expands from as little as a few hundred in those with smaller genome sizes, including *Arabidopsis*, to hundreds of thousands in their larger genome counterparts (e.g. maize, *Triticum*, *Hordeum*) (Bennett and Leitch 2005). Comparison studies suggest that the same general TE types are found in all plant species, however the relative proportions of diverse classes and subclasses can differ dramati-

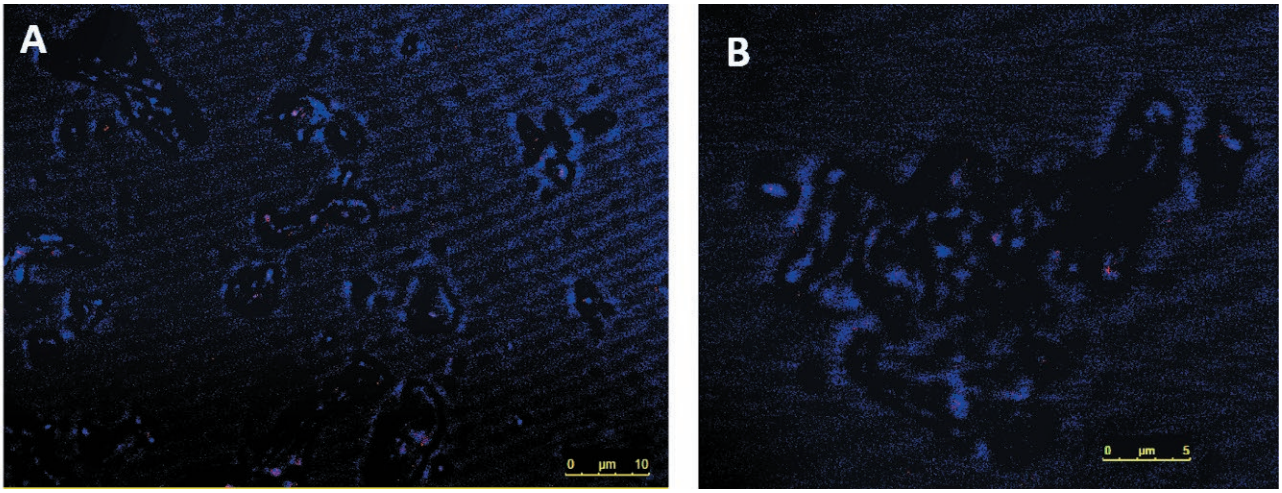

Figure 1. Display of *Sukkula internal-domain* distributions in barley root preparations *via* FISH.

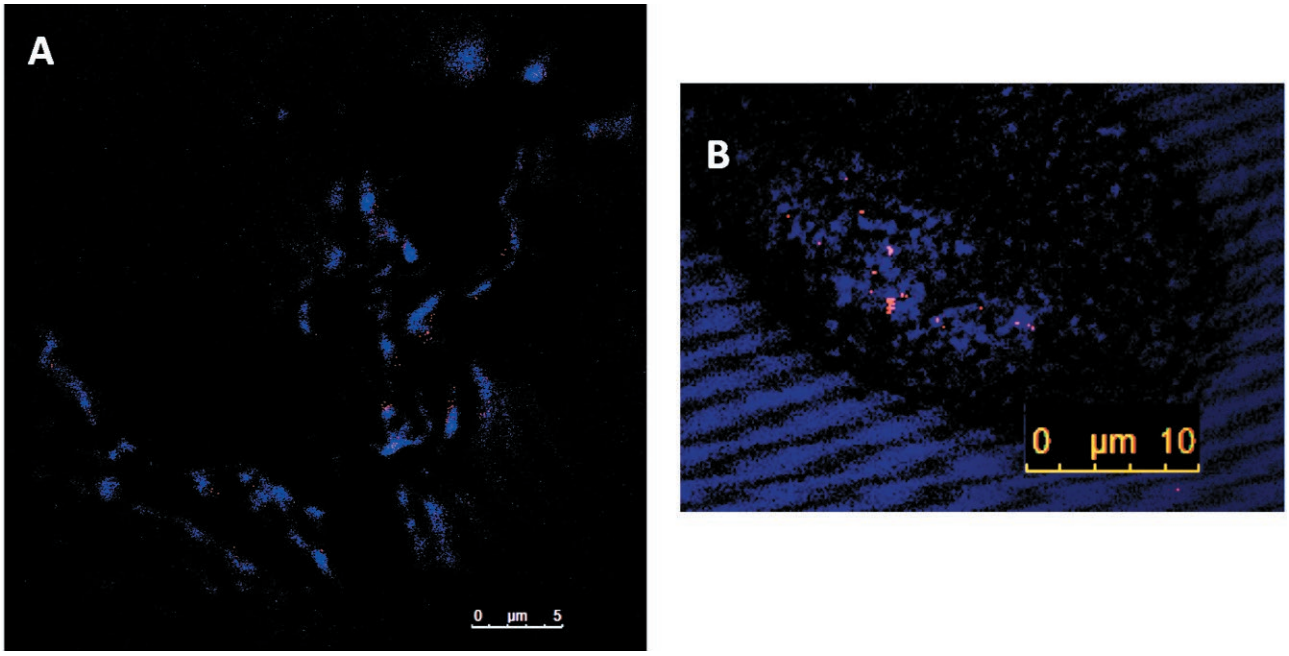

Figure 2. Display of *Sukkula LTRs* distributions in barley root preparations *via* FISH.

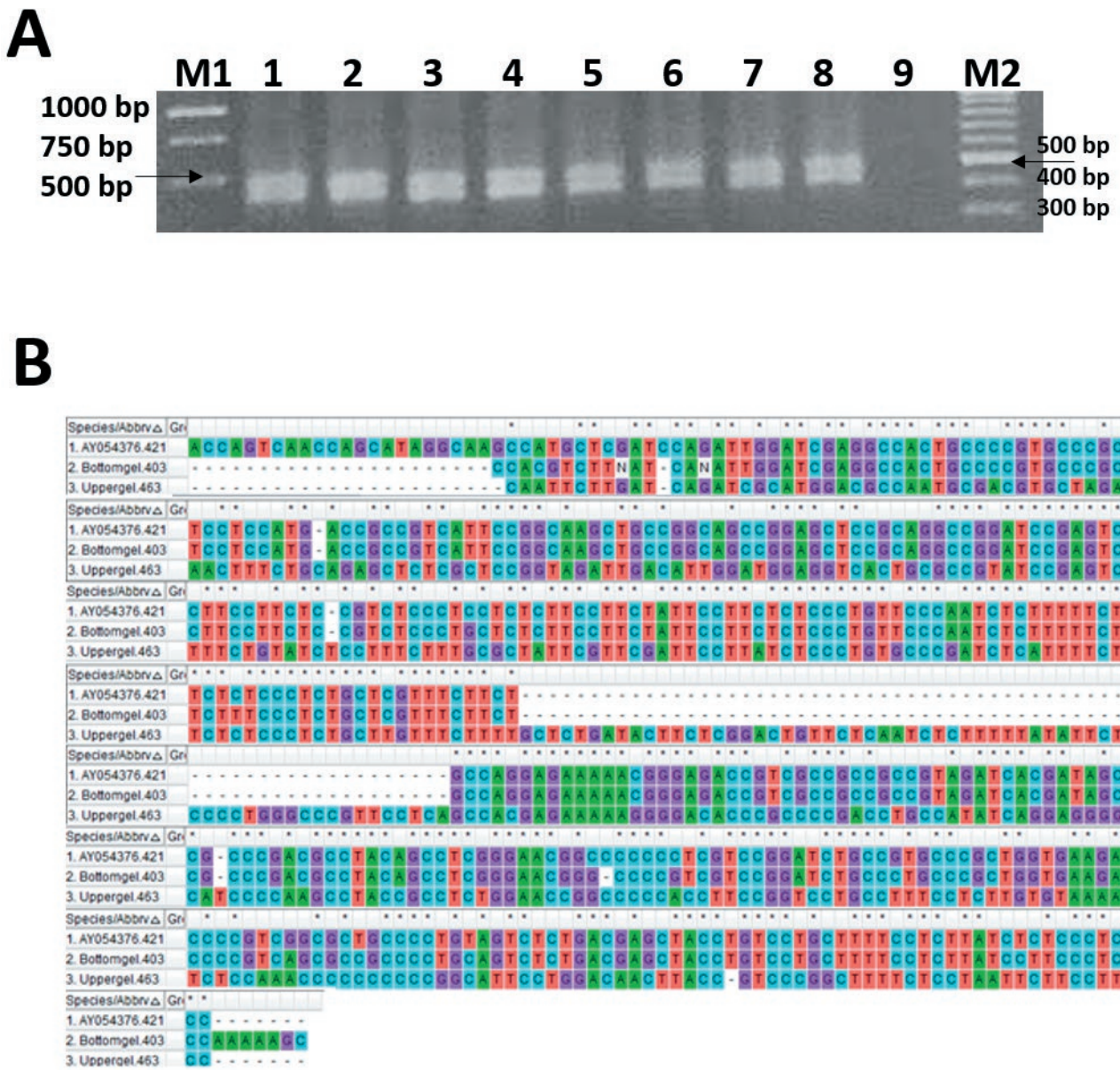

Figure 3. A) Gel figuration of sequencing primers results. **B)** Demonstration of sequencing analysis results. Original Genbank sequence AY054376 compared with the sequences recovered from bottom gel band and upper gel band displayed in Figure 3A.

cally (Kejnovsky *et al.* 2012). Moreover, LTR retrotransposon turnover in monocots have been demonstrated to be extremely rapid with both gains and losses of TEs comparing with eudicots (Ma *et al.* 2004; Vitte and Bennetzen 2006). In the present study, distribution of *Sukkula LTRs* and *intern-domain* were observed on *Hordeum vulgare* L. cv. Hasat root tips preparations using FISH analysis (see Figure 1 and 2). Because of the large genome size of barley, single-copy probes are mostly designed from BAC or YAC contigs (Vicient *et al.* 1999; Acevedo-Garcia *et al.* 2013; Bustamante *et al.* 2017). In the current study, we used direct PCR products derived from barley genomik DNA to generate single-stranded probes were short, ~ 421 bp for *LTRs* and 451 bp for *internal-domain* which were highly specific and stable for hybridization, therefore it leads to very good amplification of the signals. In addition to display of *Sukkula* elements in barley chromosomes, our group were able to observe the distributions of *SIRE1 ENV* and *GAG* by using barley root tips via FISH (Karlik and Gozukirmizi unpublished data). Nowadays, it is

also possible to use short direct labelled-PCR products to observe lncRNAs on barley chromosomes by using FISH (Karlik *et al.* 2018).

The impact of retrotransposons especially LTR-retrotransposon proliferations and loss on genome structure and evolution of plant species have been studied in species with small- or medium sized genomes. However, large sized genomes have been reported in monocotyledonous species, including maize (2.3 gigabase pairs) and barley (5.1 gigabase pairs) (Schnable *et al.* 2009; Mascher *et al.* 2017), thus we studied with barley in the present study. *Sukkula* element is known as non-autonomous LTR retrotransposons which use other retrotransposons proteins for transposition, thus we observed that *Sukkula* elements (*LTRs* and *internal-domain*) distributed on whole barley genome (see Figure 1 and 2). However, some studies demonstrated the prevalence or eventual decay of TEs in the different genomic regions depends on the process of selection and "host control" in a very long evolutionary time (Rebollo *et al.* 2012; Vitte *et al.* 2014). Kartal-Alacam *et al.* (2014) has investigated *Sukkula* polymorphism rates in non-cultured mature embryos, 40- and 80-days old callus materials by using IRAP and iPBS techniques that *Sukkula* is the second most active retrotransposon in barley genome. Moreover, our study indicated that transposition of *Sukkula* elements does not depend on the process of selection and "host control".

Kalendar *et al.* (2004) suggested that *Sukkula LTRs* are rarer than *BARE1* and not distributed in the barley genome. However, their FISH results demonstrated *Sukkula LTRs* with a high copy number. Their labelled-LTR probes were hybridized with the chromosome arms except the telomeres, nucleolar organizing regions, and centromeres, where the signals are blocking. Interestingly, we also observed a high copy number, in addition with our *LTR* and *internal-domain* probes labelled the regions in the whole chromosomes, including telomeres, nucleolar organizing regions, and centromeres (see Figure 1 and 2). However, our FISH results are consistent with the chromosome hybridization data were confirmed by PCR reaction that both *Sukkula* segments (*LTRs* and *internal-domain*) were found to be present on all barley chromosomal segments.

The abundance of repetitive DNA is mostly responsible for genome size variations in species or interspecies that especially in LTR-retrotransposons, these differences in abundance may be originated from extreme amplification through retro-transposition or from DNA loss by unequal homologous recombination, which produced solo-LTRs (Flavell 1986; Lisch 2013). During the probe synthesis, we noticed the two bands in agarose gel electrophoresis analysis (see Figure 3A). Then, we performed sequencing analysis to reveal the difference between two bands. Therefore, re-sequencing analysis of *Sukkula LTRs* has revealed that *Sukkula LTRs* had some gains, especially ~ 65 bp during the evolutionary time, indicating that this event may depend on DNA gain by unequal homologous recombination (see Figure 3B). Additionally, *Sukkula LTRs* sequences (AY054376) demonstrated 95.20% sequence identity to bottom gel band and 58.81% identity to upper gel band.

In conclusion, we were able to observe the distributions of the *Sukkula LTRs* and *internal-domain* elements via FISH by using labelled-PCR products in barley root preparations. *Sukkula* is a non-autonomous LTR retrotransposon which is still active. However, how these elements function or organize the genome is still a mystery, thus FISH analysis of TEs has important potentials to uncover the organization of large sized plant genomes.

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Karyotype analysis of *Lilium lancifolium* and four related cultivars

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Abstract. *Lilium lancifolium* is one of the most important species of genus *Lilium*. Besides ornamental value, it also has highly edible and medicinal properties. We investigated the karyotypes of *L. lancifolium* and four related cultivars. The results indicated that the ploidies of four cultivars varied from diploid to tetraploid. Both 'Flore Pleno' and 'Red Velvet' were triploid (2n=3x=36), consistenting with the wild species *L. lancifolium*. 'Sweet Surrender' was diploid (2n=2x=24), and 'Red Life' was tetraploid. All karyotypes of candidates belonged to 3B type except 'Flore Pleno', which belonged to 3A type. Karyotype symmetry analysis revealed that the wild species *L. lancifolium* had a middle value of A1, A2, and TF%, which meant that the cultivars related to *L. lancifolium* had different tendencies to symmetry compared to *L. lancifolium*, but whether they were higher or lower was unclear.

Keywords: *Lilium lancifolium*, cultivars, karyotype, chromosome.

INTRODUCTION

Genus *Lilium* includes approximately 100-115 wild species (Liang and Tamura, 2000). *Lilium lancifolium*, the tiger lily, one of the most important species of genus *Lilium*, is widely distributed in northern and eastern Asia, including Korea, Japan, China and Sakhalin (Feldmaier and McRae, 1982). Besides ornamental value, *L. lancifolium* is also extensively used as both food and a traditional Chinese medicine for many centuries in China due to its health-promoting properties and treatments of bronchitis, pneumonia, chronic gastritis (Chau and Wu, 2006; Luo *et al.*, 2012; Joung *et al.*, 2007; Kwon *et al.*, 2010; Gao *et al.*, 2015). In addition, *L. lancifolium* has been used as a modal plant to study the mechanism of bulbil formation in lilies (Yang *et al.*, 2017, 2018; He et al, 2020), because it is one of the four *Lilium* species which can bear bulbils in leaf axils (McRae, 1998; Liang and Tamura, 2000; Bach and Sochacki, 2012).

Karyomorphological investigations are very important for the views of taxonomical, ecological and cytological studies. Although the karyotype features are generally constant in a group of species and even a genus, the variations in structure and/or number can change the number, size, and position of the centromere on chromosomes, causing genetic variation (Ahn *et al.*, 2017). In addition, chromosome number can also complement information of polyploidy and other highly significant genome changes which are invisible by morphological and molecular methods. For more than 40 years, karyotype analysis of many wild *Lilium* species including *L. lancifolium* (Gill *et al.*, 1974; Vosa *et al.*, 1976; Gao *et al.*, 2011) and hybrids (Khan *et al.*, 2009; Liu *et al.*, 2011) has been carried out. That provides a lot of significant theoretical basis for cytological and taxonomic studies of *Lilium*. It is interesting that all *Lilium* species are diploids (2n=2x=24) except *L. lancifolium*, which consists of ploidy complex with diploids, narrowly distributed, and triploids (2n=3x=36), widely distributed in inlands of China, Korea, Japan and Russia, in nature (Noda 1978, 1986; Kim *et al.*, 2006; Hwang *et al.*, 2011). Because *L. lancifolium* distributed in China is natural triploid (2n=3x=36) (Noda, 1986), it is highly sterile in cross breeding, neither as male or female parent, which seriously stunts the upgrading of its varieties.

Recent years, several varieties related to *L. lancifolium* have emerged on market, but the karyotype analysis of *L. lancifolium* related cultivars have never been reported. Here, we collected four cultivars related to *L. lancifolium* and investigated the karyotypes of *L. lancifolium* and four related cultivars, providing cytological and genetic foundation for the breeding of *L. lancifolium*.

MATERIALS AND METHODS

Plant materials

L. lancifolium and four related cultivars ('Red Life', 'Sweet Surrender', 'Red Velvet', 'Flore Pleno') were used as materials in this study (Figure 1). *L. lancifolium* bulbs were harvested from our farm (Beijing, China: 116.58°E; 40.07°N) in October 2017. Bulbs of *L. lancifolium* related cultivars were purchased from Licai Garden Co., Ltd (Zhejiang, China). All bulbs were planted in the matrix of peat, vermiculite, perlite with volume ratio of 5:3:1, in a greenhouse at the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing, China in March 2018. Root tips were obtained from each population for squashing.

Chromsome preparation and observation

Actively growing root tips of each populations for the length around 0.5 cm were taken between 9 am and 11 am in a clear day, and pretreated with 0.7% cycloheximide solution at 4°C for 10 h, then fixed in Carnoy's I Fluid (methanol: glacial acetic acid = 3:1) for 24 h at room temperature, and finally kept in 70% alcohal until use. The roots were hydrolyzed in 1 mol/L HCl at 60°C for 10 min, then the chopped root tips were stained in Carbol fuchsin stain for 5~6 min at room temperature. The roots must be rinsed in distilled water for 3 to 5 times before each step. The observations of the best metaphase plates were made using an Olympus CX31 (Olympus light microscope, Tokyo, Japan) equipped with a 100 × /1.25 oil objective and a mounted Canon 550D digital camera (Canon, Japan).

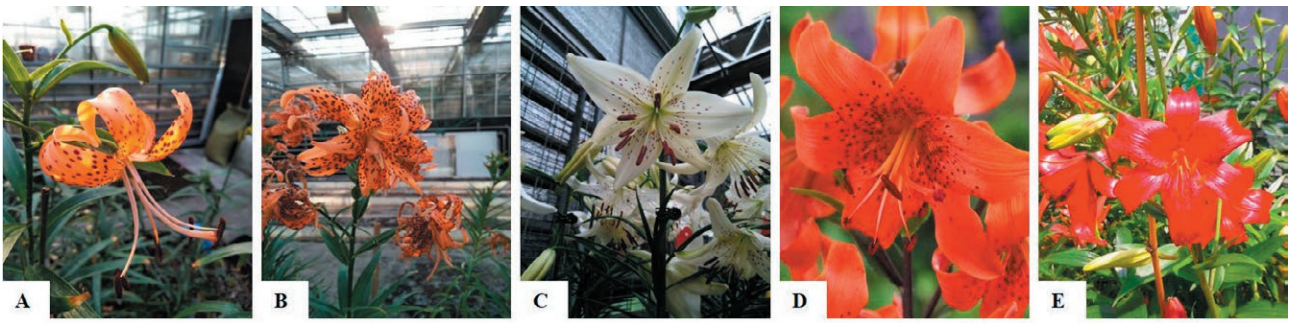

Figure 1. The five materials in this study. A: *L. lancifolium*, B-E: *L. lancifolium* cultivars: 'Flore Pleno', 'Sweet Surrender', 'Red Life', 'Red Velvet', respectively.

Karyological analyses

The measurement of basic parameters such as long arm (LA), short arm (SA) related to every chromosome was performed utilizing Photoshop V.7. Based on which we calculated total length of genome (TLG), AR (arm ratio), CI (centromeric index), LA% (long arm percentage), SA% (short arm percentage), TF% (total form percentage), VRC (value of relative chromatin), A1 (intrachromosome asymmetry index), A2 (interchromosome asymmetry index) and DI (dispersion index). The Karyotype formula was calculated according to the definition of metacentric (m), submetacentric (sm), telocentric (t), and sbtelocentric (st), proposed by Levan *et al.* (1964). The karyotype classification was defined by using the method of Stebbins (1971). Finally, the data analysis was conducted in excel.

RESULTS

This study revealed detailed pictures of mitotic chromosome plates, related karyotypes and karyograms in *L. lancifolium* and its four cultivars (Figure 2). The related parameters and karyotypic formula were summarized in Table 1 and Table 2.

The results showed that, *L. lancifolium* (Figure 2-A) is triploid, which had a chromosome number of 2n=3x=36; while in the four cultivars, only 'Flore Pleno' (Figure 2-B) and 'Red Velvet' (Figure 2-E) were triploid, with the same chromosome number as *L. lancifolium*, 2n=3x=36; 'Sweet Surrender' (Figure 2-C) was diploid, 2n=2x=24; and 'Red life' (Figure 2-D) was tetraploid, 2n=4x=48. In five materials, only *L. lancifolium* had satellites attached to the first two pairs of chromosomes, and 'Flore Pleno'had one group of chromosomes attached with satellites, the other three cultivars had no satellites. According to the karyograms in Figure 2, besides 'Red Velvet' had two groups of telocentric chromosome, *L. lancifolium*, 'Flore Pleno' and 'Sweet Surrender' had one set telocentric chromosome, and 'Red life' has no telocentric chromosome (Figure 2).

The length of shortest chromosome in *L. lancifolium* (Table 1-A) is 10.27 μm, which was obviously longer than its four cultivars'. The total genome length ranged from 117.44 to 191.05 μm between tetraploid 'Red Life' (Table 1-D) and wild species *L. lancifolium* in all populations. Even the karyotype formulas of five materials were complicated, at least four different chromosome-types were contained in each species, most of the five materials had the same karyotype 3B except 'Flore Pleno' (Table 1-B), whose belongs to 3A (Table 1). The

Table 1. Karyotype analysis of *L. lancifolium* and four related cultivars.

Sample	Chromosome number	Chromosome size range (μm)	Total genome length (μm)	Karyotype	Karyotypic formula
A	2n=3x=36	10.27-21.15	191.05	3B	3m(2SAT)+3sm(3SAT)+12st+15t+3T
B	2n=3x=36	7.57-14.80	127.37	3A	3sm(3SAT)+18st+12t+3T
C	2n=2x=24	8.68-19.83	155.29	3B	2m+4sm+4st+12t+2T
D	2n=4x=48	6.52-14.15	117.44	3B	4m+8sm+28st+8t
E	2n=3x=36	7.25-16.54	153.44	3B	3m+9sm+18st+6T

Notes: A: *L. lancifolium*, B-E: *L. lancifolium* cultivars: 'Flore Pleno', 'Sweet Surrender', 'Red Life', 'Red Velvet', respectively.

Table 2. Mean of parameters of chromosomes analysis of *L. lancifolium* and four related cultivars.

Species	TL	LA	SA	AR	CI	LA%	SA%	TF%	VRC	A1	A2	DI
A	15.64	12.98	2.66	4.88	0.17	6.79	1.39	16.70	15.92	0.79	0.20	2.47
B	10.45	8.92	1.53	5.83	0.15	7.00	1.20	14.45	10.61	0.81	0.21	3.01
C	12.94	10.49	2.45	4.28	0.19	6.67	1.58	18.95	12.94	0.77	0.26	3.09
D	9.78	7.58	2.20	3.45	0.22	6.45	1.87	22.50	9.79	0.71	0.22	3.44
E	12.79	10.06	2.73	3.68	0.21	6.56	1.78	21.35	12.79	0.70	0.19	3.43

Notes: A: *L. lancifolium*, B-E: *L. lancifolium* cultivars: 'Flore Pleno', 'Sweet Surrender', 'Red Life', 'Red Velvet', respectively. TL: total length of chromosome, LA: long arm, SA: short arm, AR: arm ratio, CI: centromeric index, LA%: long arm percentage, SA%: short arm percentage, TF%: total form percentage, VRC: value of relative chromatin, A1: intrachromosome asymmetry index, A2: interchromosome asymmetry index, DI: dispersion index.

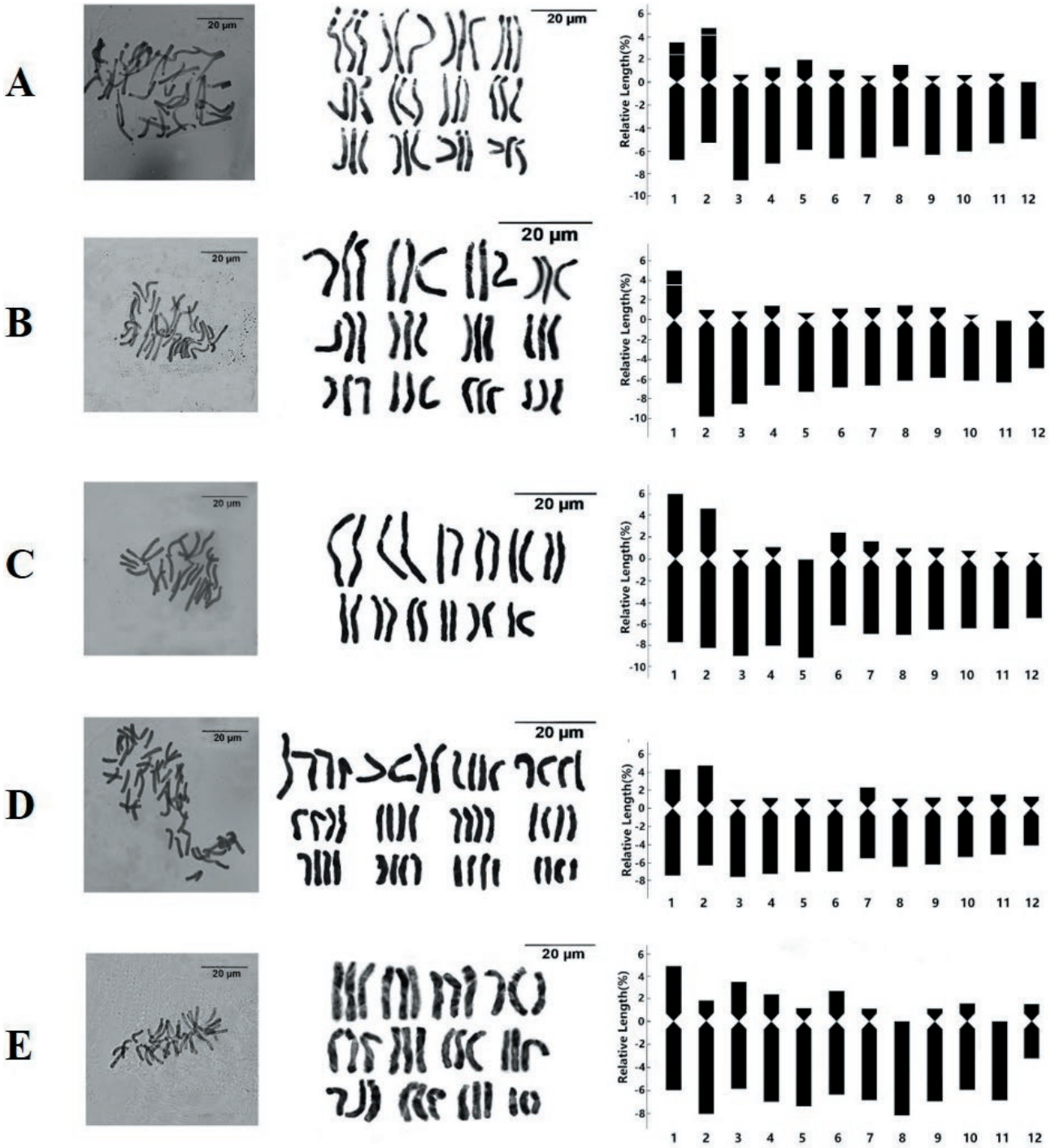

Figure 2. Mitotic chromosome plates, related karyotypes and karyograms of *L. lancifolium* and four related cultivars. A: *L. lancifolium*, B-E: *L. lancifolium* cultivars: 'Flore Pleno', 'Sweet Surrender', 'Red Life', 'Red Velvet', respectively.

mean value of the chromosome long arm varied from 7.58 μm to 12.98 μm in 'Red life' (Table 2-D) and *L. lancifolium* (Table 2-A), respectively. The average of short arm lengths ranged between 1.53 μm and 2.66 μm in 'Flore Pleno' (Table 2-B) and *L. lancifolium*. And the average total length of chromosomes varied from 9.78 μm to 15.64 μm in 'Red life' and *L. lancifolium* (Table 2).

To evaluate the symmetry of karyotype, we calculated AR (arm ratio), CI (centromeric index), LA% (long arm percentage), SA%(short arm percentage), TF% (total form percentage), VRC (value of relative chromatin), A1 (intrachromosome asymmetry index), A2 (interchromosome asymmetry index) and DI (dispersion index) respectively based on TL (total length of chromosome), LA (long arm) and SA (short arm) (Table 2). The results showed that, among the studied populations, the highest TF% value (22.50) was estimated in 'Red Life' and the lowest TF% value (14.45) was estimated in 'Flore Pleno', the TF% valve (16.70) of *L. lancifolium* was the second lowest. The analysis of the intra-chromosome asymmetry (A1) and inter-chromosome asymmetry (A2) revealed that, 'Red Velvet' (with mean value of A1=0.70, A2=0.19) presented the smallest asymmetry. In this study, the wild species *L. lancifolium* had a lowest DI value (2.47), and the DI values four cultivars were much higher (3.01~3.44) (Table 2).

DISCUSSION

The karyomorphological investigations of *Lilium* are very important for the views of taxonomical and ecological studies (Ahn *et al*., 2017). In this study, the results showed that, *L. lancifolium* (A) was triploid, which had a chromosome number of 2n=3x=36, coinciding with previous report (Gao *et al*., 2011). While the ploidy of the four cultivars related to *L. lancifolium* (A) varied from diploid to tetraploid. Since the chromosome numbers of F1 hybrids of triploid *L. lancifolium* × diploid *L. leichtlinii* range from 24 to 34 (Suzuki and Yamagishi, 2016), and the chromosome numbers of F1 hybrids of triploid *L. lancifolium* × tetraploid 'Brunello' can reach to 50 (Ma, 2017), these four cultivars we studied might be obtained by hybridization between triploid *L. lancifolium* and other tetraploid lilies. According to previous studies, all the karyotypes of *Lilium* belong to 3B and 3A type (Stebbins, 1971; Gao *et al*., 2011). In our study, only 'Flore Pleno' belonged to 3A, the other four belonged to 3B. That corroborate that the karyotype of genus *Lilium* is stable.

Both the karyograms and the value of TF% indicate that the karyotypes of five materials are very asymmetric. By using the values of A1, A2 (Zarco, 1986) and TF% (Huziwara, 1962), we can evaluate the symmetry of karyotypes among close classes. The present study revealed that the wild species *L. lancifolium* had a middle value whether of the A1, A2, or TF%, which meant that the cultivars related to *L. lancifolium* had different tendencies to symmetry compared to *L. lancifolium*, but whether they were higher or lower was uncertain.

DI index plays an important role in arranging the species within the same class of karyotype asymmetry in an advancing order of specialization by permitting further gradations, as depicted by species arrangement within sections (Lavania and Srivastava, 1992). We found that the wild species *L. lancifolium* had the lowest DI value compared to its cultivars. This might indicate that the hybrid progenies of *L. lancifolium* tend to have higher DI values.

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An adverse effect of meiotic abnormalities on spore fitness in medicinal fern *Glaphyropteridopsis erubescens* (Wall. ex Hook.) Ching

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Abstract. *Glaphyropteridopsis erubescens* plants were collected from Parvati valley, Himachal Pradesh, India. Meiotic investigations in the accessions collected from different areas of Himachal Pradesh revealed normal meiosis and good spore fertility. However, the accessions collected from Parvati valley has depicted meiotic anomalies and high spore abortion index. All populations shared the same cytological status of $2n = 72$, which is in line with previous records. Individuals depicted normal behavior at diakinesis and metaphase-I but later stages were abnormal. It includes chromatin stickiness, interbivalent connections, early and late disjunction. The formation of laggards at anaphase-I lead to series of abnormalities such as chromatin bridges, random sub-grouping of chromosomes at anaphase-II, chromatin fragmentation and irregular sporogenesis. Large numbers of micronuclei were present in triads and tetrads along with pycnotic nuclei. Empty and some abnormal sporangia with heterogenous spores were also observed.

Keywords: *Glaphyropteridopsis*, abnormal, meiosis, sporogenesis, pteridophyte, Parvati valley.

INTRODUCTION

Pteridophytes are treasures of valuable secondary metabolites that helped them to withstand harsh environmental conditions. Medicinally, they are reported to have antioxidant, antimicrobial, antiviral, anti-inflammatory, antitumor and anti-HIV properties (Baskaran *et al.* 2018). Fern and fern-allies are the major medicinal resource of phenols, flavonoids, alkaloids and steroids along with some unique phytochemicals having numerous industrial applications (Shinozaki *et al.* 2008). The tribes of North West India are using crosiers, rhizomes and fronds of *Adiantum capillus-veneris*, *Actiniopteris radiata*, *Adiantum caudatum*, *Adiantum philippense*, *Adiantum venustum, Diplazium esculentum, Pteris vittata, Pteris wallichiana, Asplenium trichomanes, Asplenium nidus and Angiopteris evecta etc.* in different formulations of drugs and ointments. *Thelypteris* species have been used to

cure cold, itching, nerve pain, rheumatism, swellings, spermatorrhea, body pain, backache, cuts and wounds, bone fracture, sprain, gastric trouble, malaria fever, body pain, sprain, boils, cancer and asthma (Sureshkumar *et al.* 2018).

North West Himalayas are facing major threat to biodiversity due to habitat destruction and pollution. They are the reservoirs of medicinal ferns as they are habitat specific and needs special care if grown in nurseries and botanical gardens. Some of high altitudinal ferns are nearly impossible to grow outside their natural habitat like presently studied fern. So, evaluation of wild accessions from different parts will be helpful in finding new and stable morphotypes and chemotypes among them. The reproductive success of medicinal ferns largely depends upon spore viability. This can be influenced by meiotic behavior and sexual status of the species. Meiotic abnormalities such as chromosomal fragmentation, bridges, laggards, micronuclei and cytomixis are known to cause sterility in number of plant species. Plant chromosomes exhibit different types of aberrations caused by mutagens, radiations or temperature etc. and they acts as sensitive indicators to environmental pollutants. So, higher plant systems acts as an indicator of possible genetic damage caused by environmental mutagens (Grant 1978).

This paper deals with meiotic investigation of *Glaphyropteridopsis erubescens* (Wall. ex Hook.) Ching (=*Thelypteris erubescens* (Wall.) Ching (Thelypteridaceae), an important medicinal fern collected from disturbed areas of Parvati valley. This family comprises 20 genera and 1000 species growing in forests with ravines of low mountains, between altitudes of 800-2000m, distributed mainly in China, Taiwan, Japan, Myanmar, Pakistan, Philippines, Vietnam, Nepal and Bhutan. In India, *G. erubescens* grows well in shady habitats of Himachal Pradesh, Uttarakhand, Sikkim, Darjeeling, Arunachal Pradesh, Meghalaya and Jammu and Kashmir. Plants of *G. erubescens* are 2–3m tall, the rhizomes are stout and they are decumbent, woody and glabrous. Fronds are clustered, stipes are 1–2 m, thicker than 1 cm, ribbed, glabrous, throughout stramineous and often reddish. The pinnae are in 40–50 pairs per frond, opposite, sessile and proximal several pairs strongly oblique distally.

This fern has noteworthy medicinal potential as leaf decoction is used to treat indigestion, dough of fronds is applied externally for rheumatism and root powder is used as an antidote in scorpion bite in Nigeria (Nwosu 2002). Powder of dried rhizome mixed with rice water can be internally administered for gonorrhea, especially for leucorrhea by the people of Deoprayag area

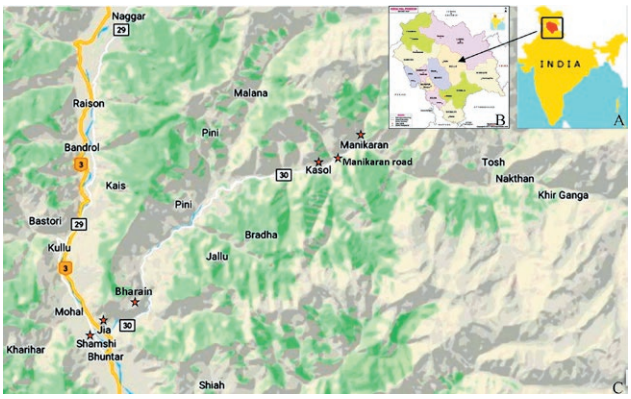

Figure 1. (a) Map showing the geographical location of Himachal Pradesh in India; (b) Map depicting various localities of Himachal Pradesh (Arrowed Kullu district of Parvati valley); (c) Localities visited for the collection of accessions from Parvati valley (highlighted with star).

in Garhwal Himalayas, India (Gaur and Bhatt 1994). *G. erubescens* is a natural source of kaempferol derivative kaempferol-3-O-α-L-rhamnoside involved in a variety of signaling pathways. Kaempferol has potential to significantly modulate a variety of signaling pathways that are induced in many adverse clinico pathological conditions (Kashyap *et al.* 2017). In the North East of India, fresh fronds of fern locally called "pire uneu" is used in the making of fermented cakes called marcha, amylolytic mixed starters similar to other regions of North East such as hamei of Manipur, pham, ipoh and phab of Arunachal Pradesh, humao of Assam and thiat of Meghalaya (Tamang *et al.* 2012).

During exploratory surveys to analyze diversity in medicinal plants of North West India, we had collected accessions from Parvati Valley (Figure 1). Its vernacular name is "Vaarne" and rhizomes were being used to prepare decoction for reproductive disorders by local people. Parvati valley is situated between 31°21'21"–32°25'0"N and 76°56'30"–77°52'20"E in South east of Kullu, altitude ranging between 1100–5550m above mean sea level. The valley is under extreme biotic and abiotic pressure due to rampant tourism, proliferation of concrete structures, construction of roads, dams, piles of garbage, hippie culture, drug trafficking (marijuana or hash) which had affected the biodiversity in recent years. All these activities result in degradation of forests and pose serious threat to fern inhabitants along with other plant communities (Sharma 2005).

Meiotic abnormalities were found to be consequence of extreme environmental factors in some species. Abortion in microspores of *Isoetes sinensis* was linked to anthropogenic activities causing habitat fragmentation

and decrease in numbers drastically (Heng-Chang *et al.* 2007). The present study aims to analyze the abnormal meiotic course and spore abortion index in this fern from disturbed locations within Parvati valley, Himachal Pradesh, India. The present work is the first attempt to study the cytology of this fern species from Parvati valley.

MATERIALS AND METHODS

The material for meiotic studies was collected from wild plants growing at various localities of Parvati valley, Himachal Pradesh, India in monsoon season (July to September). Pinnae from fronds with young sporangia were fixed in Carnoy's fixative ethanol: chloroform: glacial acetic acid (6:3:1, v/v) for 24 h at room temperature and then transferred to 70% ethanol and stored under refrigeration until use. To study meiotic course, young sporangia were squashed in 1% acetocarmine. A number of freshly prepared slides per plant were carefully examined and several spore mother cells (SMCs) were observed to determine chromosome number and frequency of meiotic abnormalities at different stages. Specimens were dried by keeping them in the folds of blotting sheets to remove excess moisture and they were supervised timely to keep them away from fungal growth or any other type of contamination. Mature spores taken onto the microscopic slide with the help of needle were treated with 1:1 glycerol and acetocarmine mixture (Marks 1954). The procedure followed for observations on number of spores per sporangium was as given by Huang *et al.* (2011). Minimum number of sporangia (8-10) was dealtwith at a time. Well stained, normal looking spores were observed for calculating average spore size and spore fertility. Distorted, deformed and poorly stained spores were taken as sterile. The spore abortion index (SAI) was calculated (Hornych and Ekrt 2017). Photomicrographs of SMCs and spores were taken from the freshly prepared slides using Magnus MLX microscope. Identification was done from fern floras (Beddome 1870; Dhir 1980 and Khullar 1994), efloras and by comparing specimens with already deposited specimens of Prof. S. S. Bir at Herbarium, Department of Botany, Punjabi University, Patiala. Finally, pressed and dried voucher specimens were deposited in the Herbarium, Department of Botany, Punjabi University, Patiala and their accession numbers (PUN) were obtained (Table 1).

RESULTS AND DISCUSSION

The cytological information for Thelypteridaceae is available for 51 species and in genus Thelypteris, there are seven basic chromosome numbers, *viz*. 36, 35, 34, 32, 31, 30 and 27 (Loyal 1963). Meiotic behavior was studied in six populations of *G. erubescens* from Parvati valley, Himachal Pradesh, India. The chromosome count of the presently investigated fern is in line with previous reports of $n = 36$ from the Himalayas (Verma and Loyal 1960; Loyal 1961; Loyal 1963; Mehra and Khullar 1980; Khullar *et al.* 1983, 1988), Nepal (Roy *et al.* 1971) and South India (Irudayaraj and Manickam 1987).

Meiosis is dynamic cellular process which maintains genome stability and integrity. Any error in meiotic process can lead to variations in chromosomal structure and ploidy level. Plant species are most acceptable material for meiotic studies because of availability of more genetic resources (Cai and Xu 2007). The accessions collected from Parvati valley depicted meiotic anomalies and high spore abortion index (**Figure 2**) while large number of populations from other parts of North West India were normal. SMCs showed a high degree of abnormalities at all stages of meiosis such as stickiness, unoriented bivalents, laggards, early and late disjunction

Table 1. Populations with altitude, voucher specimen, percentage of abnormal SMCs in *G. erubescens*.

Populations with altitude	PUN*	Meiotic abnormalities (%)							
		Stickiness	Unoriented bivalents	Laggards	Bridges	Early disjunction	Late disjunction	Interbivalent connections	Chromatin fragmentation
HP: Jia (1,125m)	4867	36.31	7.89	13.15	11.84	3.94	5.26	7.89	19.73
HP: Manikaran Road (1,423m)	4868	32.30	7.69	6.15	10.76	9.23	4.61	6.15	9.23
HP: Bharain (1,889m)	4869	25.53	12.76	10.63	6.38	-	-	-	-
HP: Shamshi (1,111m)	4870	35.71	14.28	9.52	4.76	7.14	-	9.52	-
HP: Kasol (2,619m)	4871	42.37	6.77	5.08	8.47	3.38	-	10.16	-
HP: Manikaran Sahib (1,760m)	4872	46.77	3.22	6.45	-	4.83	1.61	-	8.06

PUN*= Accession number Herbarium code of Department of Botany, Punjabi University, Patiala, HP=Himachal Pradesh, India.

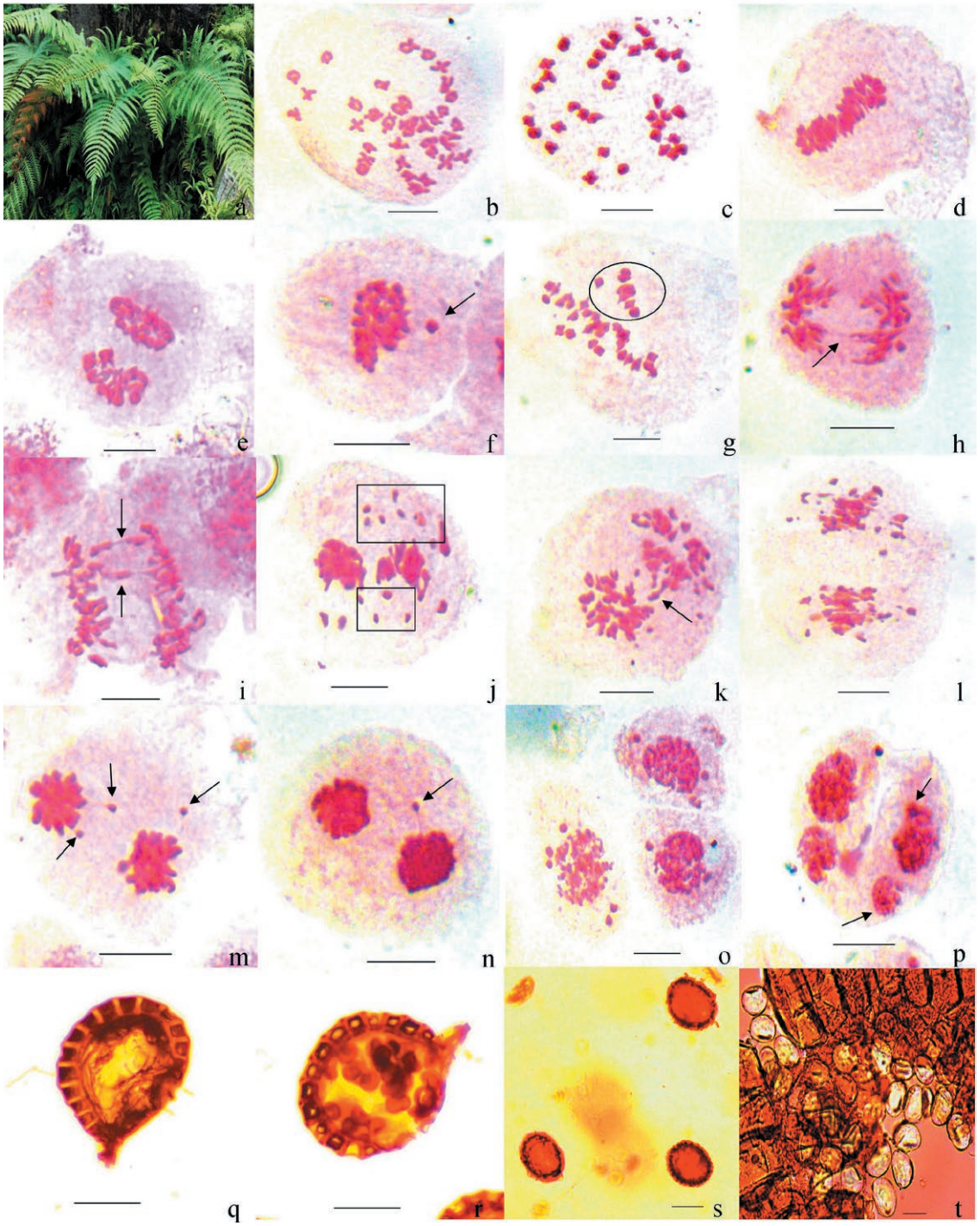

Figure 2. (a) *G. erubescens* growing wild; (b) SMC showing 36_{II} bivalents at diakinesis; (c) SMC at metaphase-I showing 36_{II}; (d) Chromatin stickiness at metaphase-I; (e) Stickiness prevailing at anaphase-I; (f) SMC with unoriented bivalent at metaphase-I (Arrow indicating one unoriented bivalent); (g) Bivalents encircled showing early disjunction at metaphase-I; (h) Arrow pointing to single chromatin bridge at anaphase-I; (i) Arrows showing multiple bridges at anaphase-I; (j) Extreme clumping of bivalents into amorphous mass with random sub grouping of chromatin material in marked area; (k) Arrow indicating laggards at anaphase-I along with agglomerisation of chromatin; (l) SMC at telophase-I with several fragments resulting from breakage; (m) Three vagrants indicated by arrows at anaphase-I; (n) Arrow showing one vagrant at telophase-I; (o) Numerous (9) micronuclei at tetrad stage; (p) Polyad with marked pycnotic nuclei; (q) Empty sterile sporangium; (r) Abnormal sporangium with varied number of spores (instead of expected 64 spores); (s) Heterogenous spores; (t) Group of sterile spores. Scale bar = 10μm.

Table 2. Sporogenesis of populations with spore abortion index and spore size.

Sporogenesis	Populations*					
	Jia	Manikaran Road	Bharain	Shamshi	Kasol	Manikaran Sahib
Number of sporads observed	98	103	84	92	106	107
Normal tetrad number (%)	45(45.91)	61(59.22)	52(61.90)	51(55.43)	60(56.60)	65(60.75)
Tetrad with micronuclei (%)	40(40.81)	31(30.09)	15(17.86)	21(22.83)	29(27.36)	20(18.69)
Tetrad with micronuclei+pycnotic nuclei (%)	8(8.16)	6(5.82)	10(11.90)	16(17.39)	12(11.32)	10(9.35)
Triad with micronuclei (%)	5(5.10)	5(4.85)	7(8.33)	4(4.25)	5(4.71)	12(11.21)
Spore abortion index	55.47±2.64	45.17±1.93	38.30±2.39	49.80±3.06	43.63±1.31	37.55±0.52
Spore size (μm)	42.42×33.42 39.51×29.57	41.85×32.38 39.74×29.01	42.72×33.83 38.76×29.02	42.56×32.26 38.71×28.97	42.35×31.64 39.86×29.65	42.23×32.09 38.67×29.82

Populations* = All the populations were collected from different localities of Parvati valley.

of chromosomes, interbivalent connections, chromatin bridges and chromatin fragmentation which leads to abnormal sporogenesis (Table 1, 2).

SMC with $n = 36$ bivalents were recorded at diakinesis (Figure 2b) and metaphase-I (Figure 2c). Chromatin stickiness prevailed at almost every stage, more prevalent at metaphase-I (Figure 2d) and anaphase-I (Figure 2e) in every population ranging from mild to severe (chromosomes appeared to be an amorphous mass with no identity) affecting 25–47% of SMCs. Chromatin stickiness can lead to serious consequences of spore sterility as these abnormalities were found to be interlinked to each other. This abnormality becomes evident at metaphase-I stage when chromosomes form intense chromatin mass but if it continues to anaphase-I obviously it will pose difficulty in bivalent separation, forming chromatin bridges. The maximum percentage of stickiness was present in population collected from Manikaran Sahib (HP) (46.77%). It has certainly affected pollen viability in many other species as they are unbalanced through irregular chromosome segregation and fragmentation (Golubovskaya 1989 and Rao *et al.* 1990). The bivalents which failed to orient themselves on the equatorial plane due to spindle abnormalities were present as unoriented bivalents (Figure 2f). The maximum percentage of unoriented bivalents was found to be present in population collected from Shamshi (HP) *i.e.* 14.28% and minimum in case of Manikaran Sahib population (3.22%). This random orientation of chromosomes and their consequent sub-grouping (Figure 2j) results due to irregular spindle activity while a single and transient spindle during mitosis and meiosis ensures the proper chromosome segregation (Caetano-Pereira and Pagliarini 2001). Interbivalents connections involving 3–4 bivalents was another phenomenon observed in four populations in which maximum percentage was in Kasol (HP) population (10.16%). These interbivalent connections were meant to keep the bivalents together before spindle formation in order to guarantee orientation of chromosomes in division plane but fusion of heterochromatic regions will result in formation of chromatic knots (Viinikka and Nokkala 1981). These diffused connections between the bivalents are associated with origin of polyploid species (Malgwi *et al.* 1997).

Early disjunction at metaphase-I (Figure 2g) and late disjunction at anaphase-I was found to be the possible reason for the presence of laggards and chromatin bridges at later stages. It has been found that late disjunction was present in three populations only ranging from 1–5%, and early disjunction *i.e.* within range of 3–9%. We came across two types of non-synchronization disjunction of bivalents early and late which arises due to different rates of terminalisation of various chromosomes of complement (Darlington 1937), changed homology of chromosomes (Koul 1971) or absence of coordination between chromosome and spindle (Sharma 1976). Early disjunction of bivalents does not affect the normal distribution of chromosomes at anaphase I but late disjunction which is more pronounced in hybrids and diploids with meiotic abnormalities will lead to bridges and laggard formation and consequently pollen malformation (Wang *et al.* 2004). This abnormal phenomenon is quite significant as it can lead to formation of gametes with numerical variations in chromosome number (Singhal *et al.* 2010). Laggards at anaphase-I (Figure 2k) are noticeable phenomenon as compared to other abnormalities involving 5–13% of SMCs, with maximum percentage in case of Jia (HP) population (13.15%). Sometimes these laggards were found to be stretched in between two poles forming single (Figure 2h) or multiple chromatin bridges (Figure 2i). Bhattacharjee (1953) observed bridge like con-

figuration which might be originated due to interlocking of chromosomes starting from prophase, persists till metaphase and during its separation at anaphase stringing out of cytoplasmic strands between them, resulting in one or two false bridge like configurations. These bridge fragment configuration is considered to be the result of crossing over in heterozygous paracentric inversions (Saylor and Smith 1966). This may lead to unequal distribution of chromosomes resulting in abortion and heterogenous pollen grains. Chromatin fragmentation resulting from breakage of chromosomes was observed at anaphase-I and telophase-I (Figure 2l) and some vagrants were observed at anaphase-I (Figure 2m) and telophase-I (Figure 2n). The possible causes of presence of laggards at anaphase were asynapsis, desynapsis, failure of chiasma formation and premature disjunction of bivalents (Gupta and Priyadarshan 1982) and abnormal spindle formation (Tarar and Dhyansagar 1980). These laggards when permanently failed to reach poles often constituted micronuclei during microsporogenesis (Jiang *et al.* 2011). One such example is the case of diploid species of *Clematis flammula* L. where meiotic abnormalities and cytomixis had resulted in pollen malformation (Kumar *et al.* 2008). Extra chromosomes were reported in case of *Ophioglossum* but in present case chromatin fragmentation were found to be present at anaphase-I and telophase-I stages, so we cannot confirm them as extra chromosomes (Goswami and Khandelwal 1980).

The incidence of meiotic aberrations (*e.g.* irregular disjunction resulting from univalent and multivalent formation) and other processes may lead to the production of non-viable spores (Ramsey and Schemske 2002). The effect of abnormal meiotic behavior on spore fertility seems to be independent of ploidy status of plant and it varies with each species. The observations regarding the effect of meiotic abnormalities on reduced pollen fertility in flowering plants have been made by Daniela *et al.* 2005; Guan *et al.* 2012; Jaryal *et al.* 2015; Kumari and Saggoo 2017; Mandal and Nandi 2017; Ramanpreet and Gupta, 2019 and Andrada 2019. Sexual species of ferns usually produced normal fertile spores whereas hybrids, apomicts, triploids etc. produce unbalanced predominantly aborted spores (Hornych and Ekrt 2017). It has been seen that if the population is seriously affected by the abnormalities, its spore abortion index will be also high. The population collected from Jia (HP) is highly abnormal with more than 75% of SMCs affected resulting in high spore abortion index (55%). Due to presence of large number of micronuclei at tetrad stage (Figure 2o), triad stage and polyad with pycnotic nuclei (Figure 2p) sporogenesis was severely affected. Empty (Figure 2q), abnormal sporangia (Figure 2r), group of sterile spores (Figure 2t) were present along with heterogenous spores (Figure 2s) leading to low spore fertility.

More investigations in row on meiotic abnormalities in different fern species will be helpful in drawing conclusions about their origin whether these are genetic, physiological or environmental. The general paradigm that can be drawn from the present observations is the fact that large percentage of meiotic abnormalities will lead to abnormal sporogenesis in this species. So, we can conclude that spore sterility may not pose problem in diploid populations unless they are seriously affected by meiotic abnormalities. These studies will be significant in studying the effect of meiotic abnormalities on reproductive behavior and calculating spore abortion index in fern species. Further, cytotaxonomic studies can solve the problems regarding scientifically correct identification of medicinal plants which is crucial for their appropriate use in pharmaceutical industry. Nowadays, conservation of medicinal plants is top most priority by conservation biologists and government agencies. Thus, evaluation of meiotic course of these plants from unexplored areas is also significant in conservation because one can opt for stable plants with high spore fertility to ensure propagation of elite germplasm.

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Toxicity of *Aristolochia* decoction: a relevant herbal in folk medicine

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Abstract. Ethnopharmacology studies report the use of *Aristolochia* (Aristolochiaceae) species as medicinal plants in various parts of the world. However, the acids aristolochic (AAs), secondary metabolites present in all species of *Aristolochia*, have cytogenotoxic activity and they are a potent carcinogen to rodents and humans. The aim of the current research was to perform to initial screening for the toxicity of *Aristolochia labiata* and *Aristolochia triangularis* decoctions through germination and growth rate, flow cytometry, mitotic index and cytogenetics analysis in *Allium cepa*. The decoctions were prepared from 2, 4, 8, 16 and 32 g L^{-1}. Decoctions at concentrations 4 g to 16 g L^{-1} significantly reduced the germination rate of *Allium cepa*. Seeds exposed to 32 g L^{-1} decoctions did not germinate. All decoctions reduced the growth rate of onion seedlings. Decoctions at 4 g L^{-1} to 16 g L^{-1} inhibited mitotic index. Highest concentrations of decoctions (8 g L^{-1} and 16 g L^{-1} for *Aristolochia labiata*; 16 g L^{-1} for *Aristolochia triangularis*) showed statistically significant increase in frequency of *Allium cepa* nuclei in the G_0/G_1 phase. Both decoctions induced the formation of heteropycnotic nuclei. Qualitative phytochemical prospecting of decocts were performed and alkaloids secondary compounds were the largest presence in both species, indicating that the AAs may be related to the observed toxicity. Caution is recommended in the consumption of decoctions from *Aristolochia labiata* and *Aristolochia triangularis* stems.

Keywords: Aristolochic acid, cytogenotoxicity, flow cytometry, heteropycnotic nuclei, *Allium cepa* test.

INTRODUCTION

Large part of the population of developing countries, especially traditional communities, depends on herbal medicine for primary health care

(WHO, 1978). The use of medicinal plants has been stimulated by Brazilian government aiming at the sustainable use of Brazilian biodiversity and the improvement of the public health system (Brasil, 2006).

Ethnopharmacology studies report the traditional knowledge on the use of *Aristolochia* (Aristolochiaceae) species as medicinal plants in various parts of the world (Heinrich *et al.,* 2009; Michl *et al.,* 2013). In Brazil, Albuquerque *et al.* (2007) verified that *Aristolochia labiata* (flowers, leaves and whole plant) is used by traditional communities in the caatinga region to relieve or cure menstrual colic and uterine inflammations. Decoction, infusion and maceration of leaves and stems of *Aristolochia triangularis* Cham. have traditionally been used to treat gynecology and urinary, gastro-intestinal, respiratory and musculoskeletal and joint diseases (Araujo & Lemos, 2015; Bolson *et al.,* 2015). The ethnobotanical survey showed that the *Aristolochia triangularis* is used as tea for the cure of diseases and/or culturally defined symptoms. Also, the species is used for the cure of spiritual diseases in the form of baths (Silva, 2008). Araujo and Lemos (2015) and Bolson *et al.* (2015) document that *Aristolochia triangularis* had the highest use values among the species cited by residents of traditional communities in the Northeast and South of Brazil, respectively.

Some medicinal plant compounds have their toxicological properties well documented in the literature. Despite the therapeutic effects of *Aristolochia* species, these plants present the Aristolochic acids (AAs) which are nitrophenanthrenes carboxylic acids. Aristolochic acids I (AAI) and II (AAII) present known cyto- and genotoxic activity and they are a potent carcinogen to rodents and humans (Chang *et al.,* 2007; Slade *et al.,* 2009; Hwang *et al.,* 2012; Bunel *et al.,* 2016; Youl *et al.,* 2020), causing a specific nephropathy associated with renal cancer (Li *et al.,* 2018; Sborchia *et al.,* 2019). As a consequence of their toxic effects, the consumption *of Aristolochia* species and its derivatives is prohibited in many countries such as Australia, Canada and United Kingdom (IARC, 2002; Neinhuis *et al.,* 2005). However, in Brazil its consumption is not regulated, being common to find dry parts of the plant in popular markets (Silva, 2008), pharmacies and stores of natural products, ready to be prepared. AAs toxicity becomes clinically significant after long periods of ingestion (Yamani *et al.,* 2015). Nevertheless, the knowledge concerning about toxic effect of decoctions of *Aristolochia* species is very scarce (Amat *et al.,* 2002).

Allium cepa test is commonly used to evaluate the toxic potential of medicinal plants and their metabolites (Akinboro & Bakare, 2007; Oloyede *et al.,* 2009). The germination rate of the seeds and the final growth of the seedlings are used to evaluate the phytotoxicity of the several compounds (Macedo *et al.,* 2008). Besides, *Allium cepa* test has also been accomplished to detect chromosomal abnormalities generated during the cell cycle (Vicentini *et al.,* 2001). This analysis is possible because *Allium cepa* has few chromosomes (2n = 16) with relatively large total length (Grant 1982). Still, *Allium cepa* test shows a similar sensitivity to other systems, like human lymphocytes (Fiskesjö 1985), and a correlation of 82% to carcinogenicity tests in rodents (Rank & Nielsen, 1994).

Flow cytometry (FCM) has broadly contributed to improve knowledge on the plants and animals' cell cycle and has been employed in the biomedical field, pharmacology, oncology and ploidy level determination (Jayat & Ratinaud, 1993). Currently, FCM applied in plants represent a very powerful tool to detect the cytotoxicity and DNA damage caused by drugs and environmental contaminants (Citterio *et al.,* 2002; Monteiro *et al.,* 2010; Andrade-Vieira *et al.,* 2012).

Considering the wide use of *Aristolochia labiata* and *Aristolochia triangularis* as medicinal plants, this study was conducted to analyze the toxicogenetic effects of their decoctions in root meristematic cells of *Allium cepa*. From the preliminary results obtained in this study, renal and testicular histopathology on mice is ongoing. These data are expected to help elucidate the effects of *Aristolochia* decoctions.

MATERIALS AND METHODS

Plant materials

Aristolochia labiata was collected in the forest garden of the Universidade Federal de São João Del-Rei (UFSJ), located in the city of São João del Rei, Minas Gerais State, Brazil. *Aristolochia triangularis* was collected in the Health Ministry located in the city of Venda Nova do Imigrante, Espírito Santo State, Brazil. A voucher specimen of the plants has been deposited at the herbarium of UFSJ, under reference number UFSJ132 (*Aristolochia labiata*) and UFSJ5571 (*Aristolochia triangularis*). Plant names were checked and updated with the Royal Botanic Gardens, Kew online website www.theplantlist.org.

Seeds of *Allium cepa* (Isla®; batch number: 774758; 90% of germination) were used as a test organism in current research because they are genetically and physiologically homogeneous (Leme *et al.,* 2008).

Preparation of decoctions

Stems of *Aristolochia labiata* and *Aristolochia triangularis* were boiled for 5 min in 1 L of distilled water (dH_2O). Considering that 8 g L^{-1} is the dosage commonly used in popular medicine (Simões *et al.,* 1995), 2, 4, 8, 16 and 32 g L^{-1} concentrations were prepared.

Qualitative phytochemical prospecting of both decoctions was carried out from the methodology proposed by Matos (1997). For this, the presence of the following metabolites was evaluated: phenols and flavonoids (sulfuric acid test), tannins (iron chloride III), saponins (foam index), coumarin (NaOH solution), alkaloids (Mayer and Wagner reagent), anthraquinone (ethyl ether + ammonia) and terpenoids (Liebermann–Burchard test).

Seed germination and seedling growth rates

To the test hypothesis that *Aristolochia* decoctions are phytotoxic (affects seed germination and seedling growth) to *Allium cepa*, onion seeds were exposed for 28 days to aqueous extracts. Culture medium MS (Murashige & Skoog, 1962) were supplemented with: 30 g of sucrose of L^{-1}, 0.4 g of L-glutamine, 0.04 g of L-cysteine, 7 g of $agar^{-1}$, pH = 5.7 ± 0.1. Culture media were autoclaved for 20 min at 121°C under 1 atm. Immediately after autoclaving, the decoction extracts were filter-sterilized and added to the tissue culture media. Colchicine at 0.025% was used as positive control (PC) and MS medium without addition of decocts as negative control (NC). In laminar flow, *Allium cepa* seeds were disinfected in 70% ethanol solution for 30 s and then with 1.5% $NaOCl_2$ solution for 20 min, and washed three times in autoclaved dH_2O for 1 min (Mursarurwa *et al.,* 2010). Fifteen seeds were inoculated in eight flasks containing 15 mL of the culture medium.

The inoculated seeds were maintained in a growth room with photoperiod 16/8 hours in light under 24 ± 1ºC. Seed germination (percentage of germinated seeds in each treatment after initial exposure) was recorded on the 28th day. In this time, the final length of the seedlings was measured for each treatment using a caliper rule.

Mitotic and chromosomal abnormalities

Onion seeds were germinated in Petri dishes lined with moistened filter paper with the different concentrations of decoctions. In Addition, dH_2O was used as negative control (NC) and the 0.025% colchicine as positive control (PC). Germination occurred in B.O.D. at 24°C for 72 h. *Allium cepa* roots with 1–2 cm were excised, fixed in ethanol: glacial acetic acid (3:1, v/v) and stored at -20°C. After 24h, the roots were hydrolyzed in 1N HCl at 25°C for 20 min, and subsequently the root meristems were stained with 2% acetic orcein, covered with foil and crushing the material. The slides were examined under a light microscope with 100x objective. Ten slides were prepared for each treatment being analyzed 500 cells/slide. Cytotoxic potential of decoctions was evaluated by mitotic index (number of dividing cells/total observed cells). Genotoxic effect was measured by the chromosomal abnormalities index (CA). Mutagenicity analysis was performed by micronuclei (MN) frequency.

Flow cytometry

The meristems of *Allium cepa* root after germination for 72 hours in a Petri dish were fixed in ethanol: glacial acetic acid (3: 1, v / v) were stored for 24 hours at -20 ° C, transferred to 70% ethanol and stored at -20°C. FCM analyzes were performed with 5 replicates for each decoction concentration and negative control (dH_2O), being three root meristems for each replicate. Root apical meristems were excised, washed 3 times for 10 min in dH_2O. Nuclei isolation and staining were performed according to the protocol proposed by Silva *et al.* (2010). Nuclei suspensions were analyzed by flow cytometry PAS® Partec flow cytometer (Partec® GmbH, Munster, Germany) after calibration of the equipment. The frequency of nuclei in G_0/G_1, S and G_2/M phases was measured to verify the cytotoxicity of the decoctions in each specific cell cycle period.

Statistical analyses

Statistical analyses were performed using the Bioestat 5.3 software. The Shapiro-Wilk test was used to verify the normality of the samples. As normality criteria were not satisfied, the non-parametric Kruskal-Wallis test with subsequent Dunn test ($p<0.05$) was performed.

RESULTS

Qualitative phytochemical analysis of decoctions is shown in Table 1. Decoctions presented phenols (detected by sulfuric acid) and alkaloids (detected by Mayer and Wagner reagents) as secondary metabolites. Since the alkaloid secondary compounds were the largest presence in both species, other metabolites were not detected.

Table 1. Qualitative phytochemical analysis of *Aristolochia labiata* and *Aristolochia triangularis*

Reagents	Metabolite class	*Aristolochia labiata*	*Aristolochia triangularis*
Sulfuric acid	Phenols	+	+
Sulfuric acid	Flavonoides	-	-
Iron chloride III	Tannins	-	-
Foam index	Saponins	-	-
NaOH solution	Coumarin	-	-
Mayer reagent	Alkaloids	+++	+++
Wagner reagent	Alkaloids	+	+
Ethyl ether + ammonia	Anthraquinone	-	-
Lieberman Burchard	Terpernóides	-	-

(-) absence; (+) presense

Table 2. Effects of decoctions of to *Aristolochia labiata* and *Aristolochia triangularis* on seed germination and seedling growth of *Allium cepa*

Species	Samples	Germination (%)	Seedling growth (cm)
Aristolochia labiata	MS	87.5	8.48 ±4.21
	Colchicine 0.025%	75.0	0.93± 0.25*
	2 g l^{-1}	87.5	1.46 ±0.92*
	4 g l^{-1}	62.5*	1.44± 0.37*
	8 g l^{-1}	62.5*	0.88±0.32*
	16 g l^{-1}	50.0*	0.67± 0.22*
	32 g l^{-1}	NG	NG
Aristolochia triangularis	MS	87.5	8.48 ±4.21
	Colchicine 0.025%	75.0	0.93± 0.25*
	2 g l^{-1}	75.0	0.53±0.19*
	4 g l^{-1}	50.0*	0.82 ±0.14*
	8 g l^{-1}	50.0*	0.45±0.50*
	16 g l^{-1}	50.0*	0.40±0.43*
	32 g l^{-1}	NG	NG

MS: negative control -culture medium (Murashige and Skoog, 1962). Colchicine 0.025%: positive control. NG: did not germinate. * Significant difference in relation to negative control ($p < 0.05$) - Kruskal-Wallis test.

Table 2 shows the effects of the decoctions of *Aristolochia labiata* and *Aristolochia triangularis* on seed germination and seedlings of *Allium cepa*. Seeds of the control group germinated satisfactorily. Decoctions of both species at concentrations 4, 8 and 16 g L^{-1} significantly reduced the germination rate of *Allium cepa*. Seeds exposed to 32 g L^{-1} decoctions did not germinate. All decoctions concentrations reduced the onion seedlings growth rate (Table 2, Figure 1A, 1B).

Cell proliferation rate was 13.34% in the control group (Table 3). Decoctions at 2.0 g L^{-1} did not promoted significant reduction of mitotic index. Decoctions at 4, 8 and 16 g L^{-1} inhibited cell proliferation in *Allium cepa* (Table 3). Changes in *Allium cepa* cell cycle

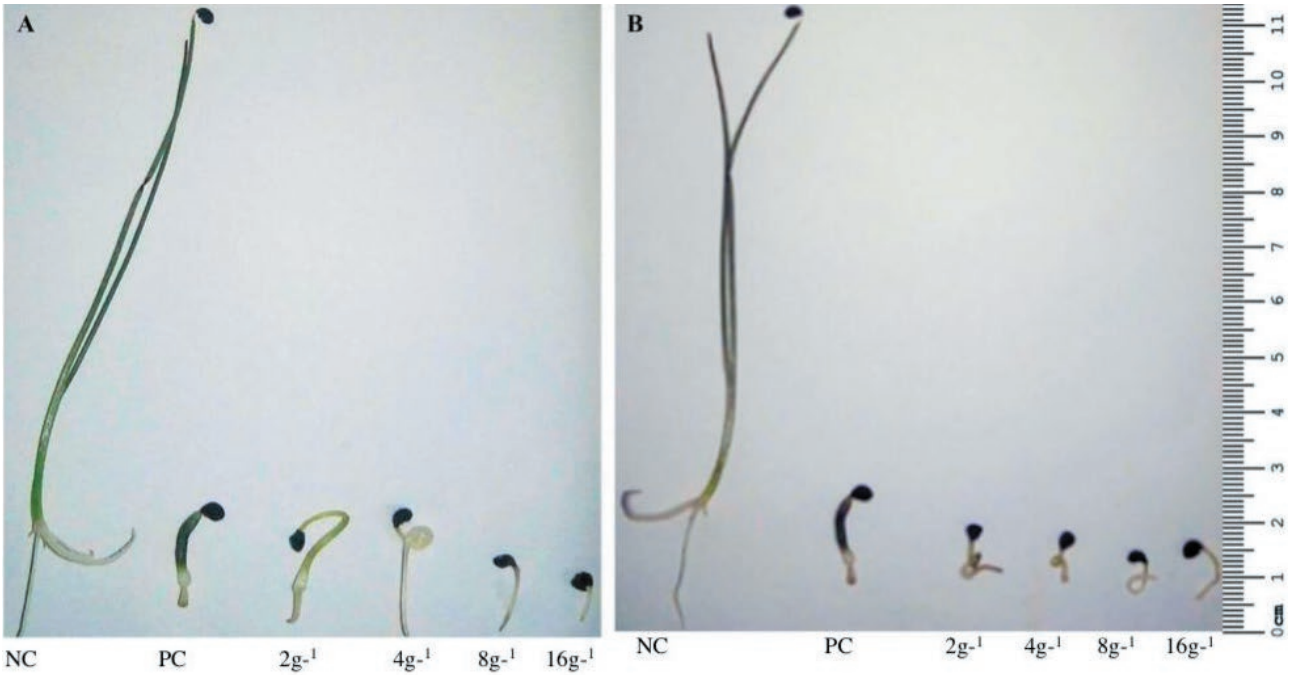

Figure 1. Seedlings of *Allium cepa* after 28 days of exposure to the decoctions of (A) *Aristolochia labiata* and (B) *Aristolochia triangularis*. NC = negative control - culture medium (Murashige and Skoog, 1962); PC = positive control (0.025% colchicine).

Table 3. Mitotic index (% percentage ± standard deviation) of *Allium cepa* root meristem cells exposed to decoctions of *Aristolochia labiata* and *Aristolochia triangularis*

Species	Samples	Mitotic index %	Number of cells in each cell cycle phase				
			Interphase	Prophase	Metaphase	Anaphase	Telophase
Aristolochia labiata	Distilled water	13.34 ± 4.72	4333	335	126	137	69
	Colchicine 0.025%	10.52 ± 3.61	4286	343	149	150	72
	2 g l^{-1}	6.92 ± 3.56	4691	75	144	81	9
	4 g l^{-1}	1.02 ± 0.86*	4949*	9*	21*	15*	6*
	8 g l^{-1}	1.10 ± 2.30*	4941*	11*	29*	13*	6*
	16 g l^{-1}	0.76 ± 0.68*	4960*	12*	6*	15*	7*
	32 16 g l^{-1}	NG	NG	NG	NG	NG	NG
Aristolochia triangularis	Distilled water	13.34 ± 4.72	4319	339	128	142	72
	Colchicine 0.025%	10.52 ± 3.61	4286	343	149	150	72
	2 g l^{-1}	8.38 ± 3.18	4951*	15	14	14	06
	4 g l^{-1}	2.20 ± 3.94*	4888*	51*	24*	24*	13*
	8 g l^{-1}	3.34 ± 3.69*	4803*	69*	73*	53*	02*
	16 g l^{-1}	3.84 ± 2.14*	4961*	16*	06*	11*	06*
	32 16 g l^{-1}	NG	NG	NG	NG	NG	NG

Distilled water: negative control. Colchicine 0.025%: positive control. NG: did not germinate.* Significant difference in relation to negative control ($p < 0.05$) - Kruskal-Wallis test.

are also shown in Table 4 and representative histograms are shown in Figure 2. According to FCM analyses, decoctions of both species promoted a concentration-dependent increase in frequency of *Allium cepa* nuclei in the G_0/G_1 phase. This increase was statistically significant for the highest concentrations of decoction (8 and 16 g L^{-1} for *Aristolochia labiata*; 16 g L^{-1} for *Aristolochia triangularis*). Thus, the frequency of nuclei in the S and G_2/M phase tended to decreased, parking cells in interphase. *Aristolochia labiata* decoctions at 8 and 16 g L^{-1} caused a greater number of cell damage; the highest dose

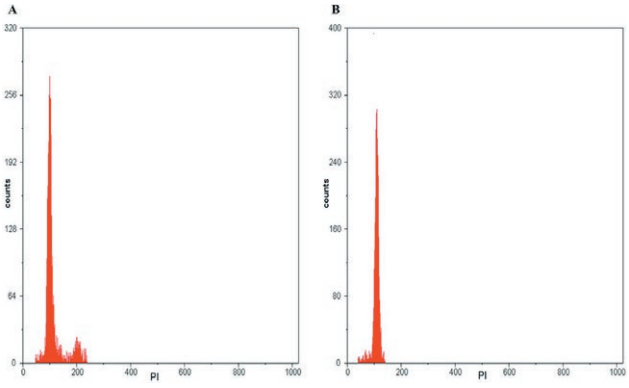

Figure 2. Histogram representative of the meristem of *Allium cepa*. A) Histogram representing the negative control. Note the presence of the G_0/G_1 peak (channel 100) G_2/M (channel 200), and between the particles at different times is S phase of the cell cycle, showing that the negative control has particles at all stage of G_0/M. B) Histogram representing the treatment of 16 g l^{-1} of *Aristolochia labiata*, observe particles in the peak (channel 100) demonstrating that the cells are in G_0/G_1, but there is no peak at (channel 200), which confirms the no progression of nuclei for the G_2/M phase, indicating that the extracts prevent cell proliferation.

Table 4. Frequency (%) of *Allium cepa* nuclei in cell cycle phases after treatment with *Aristolochia labiata* and *Aristolochia triangularis* decocts

Species	Samples	$\%G_0/G_1$	%S	$\%G_2/M$
Aristolochia labiata	Distilled water	70.08 ± 6.91	18.37 ± 4.03	11.00 ± 3.62
	2 g l^{-1}	88.96 ± 1.90	8.08 ± 1.36	3.00 ± 0.93
	4 g l^{-1}	91.34 ± 2.93	6.46 ± 2.63	2.00 ± 1.07
	8 g l^{-1}	97.16 ± 2.91*	2.57 ± 2.81*	0.27 ± 0.60*
	16 g l^{-1}	99.85 ± 0.32*	0.14 ± 0.32*	0.00 ± 0.00*
	32 g l^{-1}	NG	NG	NG
Aristolochia triangularis	Distilled water	74.08 ± 4.52	11.02 ± 4.17	14.89 ± 1.93
	2 g l^{-1}	84.72 ± 2.83	8.82 ± 1.86	6.46 ± 4.54
	4 g l^{-1}	86.23 ± 3.61	8.12 ± 1.47	5.63 ± 3.24
	8 g l^{-1}	87.18 ± 4.03	7.22 ± 2.84	5.59 ± 1.48
	16 g l^{-1}	92.28 ± 2.09*	4.34 ± 1.17*	3.38 ±1.24*
	32 g l^{-1}	NG	NG	NG

Distilled water: negative control. NG: did not germinate. * Significant difference in relation to negative control ($p < 0.05$) - Kruskal-Wallis test.

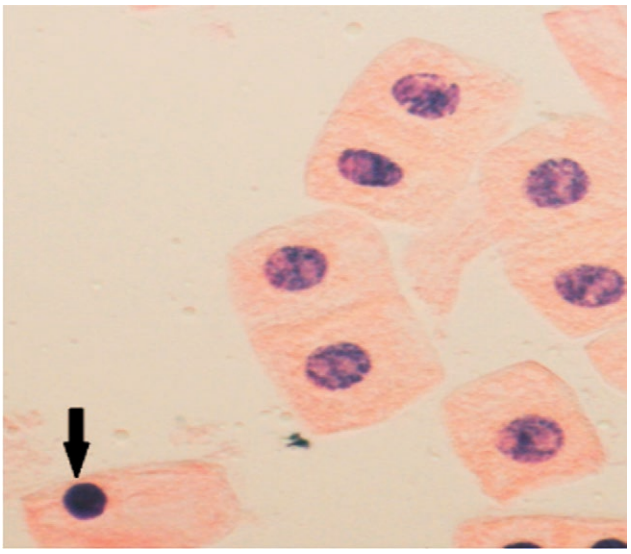

Figure 3. Heteropicnotic nucleus (arrow) in *Allium cepa* meristematic root cells after exposure to the decoctions of *Aristolochia labiata*. 1000x magnification.

Table 5. Percentage of chromosomal alterations (CA) and heteropycnotic nucleus observed in *Allium cepa* cells exposed to decoction of *Aristolochia labiata* and *Aristolochia triangularis*

Species	Samples	%CA	%Heteropycnotic nucleus
Aristolochia labiata	Distilled water	0.04 ± 0.12	0.00 ± 0.00
	Colchicine 0.025%	5.88 ± 2.81*	0.00 ± 0.00
	2 g l^{-1}	0.12 ± 0.16	0.00 ± 0.00
	4 g l^{-1}	0.00 ± 0.00	8.84 ± 1.53*
	8 g l^{-1}	0.00 ± 0.00	15.8 ± 13.03*
	16 g l^{-1}	0.00 ± 0.00	29.60 ± 29.02*
	32 g l^{-1}	NG	NG
Aristolochia triangularis	Distilled water	0.04 ± 0.12	0.00 ± 0.00
	Colchicine 0.025%	5.88 ± 2.81*	0.00 ± 0.00
	2 g l^{-1}	0.00 ± 0.00	13.4 ± 7.95*
	4 g l^{-1}	0.00 ± 0.00	17.2 ± 19.57*
	8 g l^{-1}	0.00 ± 0.00	17.1 ± 13.02*
	16 g l^{-1}	0.00 ± 0.00	19.12 ± 22.2*
	32 g l^{-1}	NG	NG

Distilled water: negative control. Colchicine 0.025%: positive control. NG: did not germinate.
* Significant difference in relation to negative control ($p < 0.05$) - Kruskal-Wallis test.

reached a level of absence of core peak in G_2/M phase. *Aristolochia triangularis* decoction at 16 g L^{-1} significantly reduced the number of nuclei in S and G_2/M phase.

Due to severe cytotoxicity, chromosomal abnormalities and micronuclei were not observed. Heteropycnotic nuclei (Figure 3) were analyzed separately. Decoctions of both species at 4, 8 and 16 g L^{-1} significantly increased the frequency of this abnormality. *Aristolochia triangularis* decoction at 2 g L^{-1} also induced heteropycnotic nuclei on the *Allium cepa* root cells (Table 5).

DISCUSSION

The genus *Aristolochia* presents a wide range of physiologically active compounds classified in five main categories: terpenes, phenols, alkaloids, flavonoids and lignoids (Pacheco *et al.,* 2009). Phytochemical analyses indicated mainly the presence of alkaloids in both decoctions studied and in a lower extent, phenols. Phenols bring advantages to the plant, as they are related to attraction of pollinators and protection against herbivory among others (Piesik *et al.,* 2011). Species of Aristolochiaceae are rich in alkaloids (Schmeiser *et al.,* 2001), including AAs that have attracted intense research interest because of cyto- and genotoxic properties of AAI and AAII (Wu *et al.,* 2005; Chang *et al.,* 2007; Slade *et al.,* 2009; Bastek *et al.,* 2019).

The amounts of AAI and AAII in decoctions were not determined. However, as the AAs are slightly soluble in water (O'Neil, 2001), we believe that possibly they are present in decoctions, which is the way by which people consume species of *Aristolochia*. Hwang *et al.* (2012) quantified AAs in aqueous extracts of *Aristolochia manshuriensis* Kom. The genotoxicity of the extracts was detected by bacterial reverse mutation assay and micronucleus in mice bone marrow erythrocytes. According to the authors, the genotoxicity of *Aristolochia manshuriensis* is directly related to the AAs.

Allium cepa test has been used as first cytogenotoxic screening of medicinal plant extracts (Dias & Takahashi, 1994; Fachinetto *et al.,* 2007; Meneguetti *et al.,* 2014; Mendes *et al.,* 2012) because the results are reliable and similar to those performed with in mammals (Rank & Nielsen, 1994), contributing to the safe use of these herbs (Mendes *et al.,* 2012).

Studies concerning toxicological activity of *Aristolochia* extracts are very scarce, in spite of their use in several countries (Amat *et al.,* 2002). In the current research, the toxic effects of decoctions of *Aristolochia labiata* and *Aristolochia triangularis* on *Allium cepa* were evaluated. Decoctions of both species at 32 g L^{-1} were phytotoxic because prevented the germination of onion seeds. The other concentrations tested promoted delayed germination and growth and mitodepressive

effects on *Allium cepa* root cells. Akinboro and Bakare (2007) also documented a relationship between macroscopic and microscopic parameters for *Allium cepa* root cells exposed to toxic aqueous extracts herbs. The growth inhibition of the seedlings was always accompanied by the reduction of the number of cells in division. Corroborating our results, Gatti *et al.* (2004) showed that extracts of *Aristolochia esperanzae* Kuntze delayed seed germination and root growth of *Lactuca sativa* L. and *Raphanus sativus* L. According to Baličević *et al.* (2015), extracts of *Aristolochia clematitis* reduced the germination and growth of *Tripleurospermum inodorum* L. (weeds), and the concentration of 100 g L^{-1} inhibited the germination of seeds of these plants. Aqueous extract at 25 g L^{-1} of *Aristolochia triangularis* presented antimitotic action to meristematic cells of *Allium cepa* (Amat *et al.,* 2002). Watanabe *et al.* (1988) documented that AAs are potent inhibitors of seed germination.

FCM analyses also showed a presence of compounds in the decoctions that delayed the progression of the cell cycle. The decoctions tested caused a concentration-dependent increase of *Allium cepa* nuclei in G_0/G_1. Consequently, *Aristolochia* decoctions promoted a reduction of *Allium cepa* nuclei in S and G_2/M phases. These results could reflect the activation of G_0/G_1 checkpoints in response to DNA damage. Plant cells have a p53-independent control of proliferation (Pelayo *et al.,* 2003). The checkpoint pathways transduce antimitogenic signals that lead to the temporary interruption of the cycle. Thus, the repair mechanisms can act before the irreversible transition to the subsequent cycle phase (Pelayo *et al.,* 2003; Junqueira & Carneiro, 2012). Also, heteropycnotic nuclei were observed in response to decoctions exposure. These markers of cell death are characterized by condensation of the nucleus (Andrade-Vieira, *et al.,* 2012), making it inoperative for failure of the enzyme synthesis (Manjo & Joris, 1995; Levin *et al.,* 1999). The activation of cell death mechanisms is the last resource to avoid proliferation of cells containing abnormal DNA. In this way, we could infer that DNA damage was not repaired in G_1, and in response, cell death pathways were activated.

Some studies also documented that *Aristolochia* extracts and AAs promoted cell cycle arrest and cell death in mammalian cell lines. Li *et al.* (2006) reported that AAI may cause DNA damage and cell cycle delay in porcine proximal tubular epithelial cell lines through a wild-type p53-independent pathway, prior to apoptosis or necrosis. Chang *et al.* (2007) found that cell cycle distribution determined by flow cytometry showed an increase of human urinary tract epithelium cells in the G0/G1 phase after exposure to AAs mixture (41% AA I and 56% AA II). Proteins levels that block the cell cycle (p53, p21 and p27) have increased. Additionally, there was a decrease in cyclinD1/cdk4 complex, which control proteins required for the progression of the cycle (Chang *et al.,* 2007). Li and Wang (2013) verified that methanol extract from *Aristolochia debilis* Siebold & Zucc. stems inhibited proliferation of human colon cancer cells by inducing sub-G_1 arrest. The authors also showed that *Aristolochia debilis* induced apoptosis in HT-29 cells by upregulation of Bax and corresponding downregulation of Bcl-2 expression as well as ROS production.

Allium cepa test was suitable for screening initial toxicity of *Aristolochia labiata* and *Aristolochia triangularis* decoctions. Our studies report similar results for other test systems. In vivo research on renal and testicular histopathology of decoctions in mice is ongoing.

CONCLUSION

Allium cepa test was used to evaluate decoctions from *Aristolochia labiata* and *Aristolochia triangularis* stems. Phytochemical analysis indicated mainly the presence of alkaloids in both decoctions studied. The decoctions promoted inhibition of onion seed germination and seedling growth. The mitodepressive effect of both decoctions was determined by mitotic index and FCM analyses. The induction of heteropycnotic nuclei suggests that decoctions promote cell death. We suggest that the AAs may be related to the observed toxicity. Caution is recommended in the consumption of decoctions from *Aristolochia labiata* and *Aristolochia triangularis* stems.

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Physical mapping of 45S and 5S rDNA in two *Sprekelia formosissima* cytotypes (Amaryllidaceae) through Fluorescent *In Situ* Hybridization (FISH)

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Abstract. Chromosome number and position of rDNA were studied in plants of *Sprekelia formosissima* (Amaryllidaceae) collected in two populations with different ploidy level (2*n*=2*x*=60 and 2*n*=5*x*=150). The 5S and 45S rRNA *loci* were localized and physically mapped using two-color fluorescence in situ hybridization probes. The diploid (2*n*=2*x*=60) cytotype showed four *loci* for the 45S rDNA in two chromosome pairs (11 and 25) in telomeric position. The 5S rDNA was present in six *loci* of three homologous chromosome pairs (3, 13 and 19) in subtelomeric and telomeric positions. The chromosomes of the pentaploid cytotype (2*n*=5*x*=150) showed five *loci* for the 45S rDNA in telomeric position and five *loci* for the 5S rDNA in subtelomeric position. The karyotypic formula is 13m + 16sm + 1 st and the karyotype symmetry/asymmetry index is TF % = 34.67, AsK % = 65.32 and Syi % = 54.81, concluding that it is an asymmetric karyotype, bimodal with one distinctively large pair of chromosomes (10.42 μm) and a gradual decrease in the size of the other chromosome pairs, from the longest of 6.84 μm, to the shortest of 2.61 μm.

Keywords: bulbous genus, karyogram, karyotypic formula, ornamental, plant chromosomes, ploidy level.

INTRODUCTION

Sprekelia Heist, is a bulbous genus of the monocotyledonous family Amaryllidaceae, it is a perennial, herbaceous monotypic genus commonly known as Aztec lily or Jacobean lily represented by the sole species *Sprekelia formosissima* (Flory 1977; Sánchez 1979); it is distributed through Mexico and Guatemala (López-Ferrari and Espejo-Serna 2002). Its scarlet flowers with curved petals have made of it an exceptional ornamental pot plant.

The technique of Fluorescent *In Situ* hybridization (FISH) has become a very useful tool for the detection of specific DNA in the genome of organisms. In this technique, the genes encoding the 5S ribosomal RNA (rDNA

5S) and the 18S-5.8S-26S ribosomal RNA (45S rDNA) are commonly used as markers for the physical mapping of plant chromosomes due to the high number of their repeating units, specific position on chromosomes and highly conserved sequences (Liu and Davis 2011). The 45S ribosomal RNA genes are clustered in tandem arrays of repeating units of the genes 18S, 5.8S and 26S, internal transcribed spacer sequences (ITS) and external non-transcribed spacer sequences (NTS), with an approximate size of 7.5-18.5 Kb in plants (Mizuochi *et al.* 2007). The 5S ribosomal RNA genes are also found in tandem repeats with an approximate size of 0.2-0.9 Kb, with a highly conserved region (120 bp long) separated by a NTS sequence (Specht *et al.* 1997). Physical mapping of rDNA has been performed in many plants such as: maize (Li and Arumuganathan 2001), orchids (Cabral *et al.* 2006), *Crotalaria juncea* (Mondin *et al.* 2007), *Agave* (Robert *et al.* 2008; Gomez-Rodriguez *et al.* 2013), asparagus (Deng *et al.* 2012), *Lilium* (Lim *et al.* 2001; Hwang *et al.* 2015), *Tigridia pavonia* (Arroyo-Martínez *et al.* 2018), among many others, however, there is little work in plants of the Amaryllidaceae family using these modern techniques of molecular cytogenetics. The objective of this study was the physical mapping of rDNA 45S and 5S in two cytotypes (diploid and pentaploid) of *Sprekelia formosissima*.

MATERIAL AND METHODS

Plant material

Sprekelia formosissima bulbs were collected from two different populations and maintained in an *in vivo* collection at the Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco A.C. (CIATEJ). Two cytotypes were found, a diploid population ($2n=2x=60$) from the road Villa-Corona-Cocula, and a pentaploid population ($2n=5x=150$) from a private collection in Zapopan, both in the state of Jalisco, México. The bulbs were placed in a container with moist substrate composed by a mixture of Peat Moss and Vermiculite (7:3) for the bulbs to produce roots. Sampling was performed in ten bulbs of each of the two populations.

Chromosome spreads

Obtaining metaphasic chromosomes was performed as described by Rodríguez-Domínguez *et al.* (2017), in brief, the root apex were pretreated in a saturated solution of α-bromonaphthalene (0.1%) for 48 h at 4 °C and fixed in a methanol:acetic acid (3:1) solution for 24 h at 4 °C. Afterwards, the root apex were washed with milli-Q water, macerated in an enzyme mixture containing each 0.2% (w/v) of pectolyase Y23, RS cellulase and cytohelicase in 10 mM citrate buffer (pH 4.5) and incubated at 37 °C for 3 h. Later, a cell suspension was obtained by vortexing, followed by washing with distilled water, centrifuging at 10,000 rpm for 45 s, washed with methanol, then, centrifuged at 11,000 rpm during 30 s. The cell pellet was resuspended in 100 µl methanol. Finally, in a fume hood, the slides were covered with a layer of pure acetic acid and 10 µl of cell suspension were added to each slide. The slides were immediately inclined at an angle of 45 degrees, and two drops of pure acetic acid were added; the slides were exposed (facing down) to vapor from a water bath at 55 °C for 5 s, finally, a drop of pure acetic acid was added and allowed to air dry.

DNA probes

Two different clones were utilized as probe, clones pTa71 and pTa794 which contains the *EcoRI* fragment of 45S and 5S ribosomal DNA from wheat respectively (Gerlach and Bedbrook 1979; Gerlach and Dyer 1980). The bacteria were cultured for 24 hours under constant agitation (225 rpm) at 37 °C in LB medium supplemented with 0.1 mg/ml ampicillin. Isolation of wheat ribosomal DNA was performed using the kit High Pure Plasmid Isolation Kit (Roche®) according to the supplier's instructions. In brief, 4 ml of the inoculated culture medium were transferred to 15 ml tubes and centrifuged at 9,000 rpm for 1 minute, the supernatant was discarded; the bacterial pellet was resuspended in 250 µl of suspension buffer and transferred to a 1.5 ml tube. 250 µl of lysis buffer were added and mixed by inversion, then incubated for 5 min and 350 µl of binding buffer were added and incubated for 5 min on ice; followed by centrifugation at 13,000 rpm for 10 min. The supernatant was transferred to a filtration membrane and centrifuged at 13,000 rpm for 1 minute; the membrane was transferred to a new tube and 500 µl of washing buffer I was added and centrifuged at 13,000 rpm for 1 minute. The column was transferred to a new tube and 700 µl of washing buffer II was added; centrifuged at 13,000 rpm for 1 minute. The flowthrough liquid was discarded and the column was centrifuged at 13,000 for 1 minute to dry the membrane, which was transferred to a new 1.5 ml tube and 100 µl of elution buffer was added and centrifuged at 13,000 rpm for 1 minute.

Labeling the probes

Fluorescent in situ hybridization (FISH) was performed using two different probes (45S and 5S wheat rDNA) which were direct labelled as follows: 1 µg of rDNA in 12 µl mQ water, 4 µl Nick translation mix (Roche®) and 4 µl of 5x fluorophore mix (5 µl of each 2.5 mM dATP, dCTP, dGTP, 3.4 µl 2.5 mM dTTP, 4 µl 1 mM labeled dUTP and 27.6 µl mQ water) were incubated at 15 °C for 90 min. Reaction was stopped by adding 1 µl of 0.5 M EDTA (pH 8.0) and heating at 65 °C for 10 min. 45S rDNA was labeled with Tetramethylrhodamine-5-dUTP and 5S rDNA with Fluorescein-12-dUTP.

Fluorescent in situ hybridization

Slides with metaphase chromosomes were incubated at 37 °C overnight, the next day, each slide was incubated in 200 µl RNase A (100 µg/ml) in 2xSSC at 37 °C for 1 h and washed three times for 5 min each in 2x SSC, incubated in 0.01 M HCl for 2 min. 200 µl Pepsin (5 µg/ml) were added and incubated at 37 °C for 10 min, washed in mQ water for 2 min and in 2x SSC for 5 min each. The slides were incubated in 4% paraformaldehyde for 10 min and washed three times in 2x SSC for 5 min each. The slides were dehydrated in 70%, 90% and absolute ethanol series for 3 min each and air-dried. Hybridization followed using a mixture consisting of 20x SSC, 50% formamide, 10% sodium dextran sulphate, 10% SDS and 25-50 ng of each probe. The DNA was denatured by heating the hybridization mixture at 70 °C for 10 min and then placed on ice for at least 10 min. For each slide, 40 µl of hybridization mixture was used. The preparations were denatured at 80 °C for 10 min and incubated overnight in a humid chamber at 37 °C. After overnight hybridization, the slides were washed three times in 2x SSC at 37 °C for 5 min each, in the last wash temperature was increased to 42 °C and the slides were then washed three times in 0.1x SSC at 42 °C each followed by three washes in 2x SSC at RT for 5 min each. Chromosomes were counterstained with 1µg/ml DAPI (4,6-diamidino-2-phenylindole) and a drop of Vectashield anti-fade (Vector laboratories) was added for its examination under a Leica DMRA2 microscope equipped with epi-fluorescent illumination, filter sets of DAPI, FITC and Cy3. Images were captured by a Evolution QEi (Media Cybernetics) monochrome digital camera and processed with Image-Pro Plus software. The DAPI images were sharpened with a 7x7 High Gauss spatial filter. DAPI, FITC and Tetramethylrhodamine fluorescence were pseudo-colored with their respective dye tint for the FISH analyses.

Karyotype analysis

Karyotype analysis was based on at least 10 high-quality metaphase plates with almost similar degree of chromatin condensation, chromosome measurements were made using the freeware software Micromeasure (Reeves 2001) vers 3.3. The following measurements of each pair of chromosomes were made: Chromosome length, long arm (L), short arm (S), arm ratio (L/S). The chromosomes were assorted into different categories based on arm's ratio arranged according to Levan *et al.* (1964) and the karyogram was performed; the chromosome homology was assigned according to similarities in length, morphology and centromere position. The number of homologous chromosomes was assigned sequentially according to the reduction of the chromosomal length and in base to centromere position. Three different methods of evaluating karyotype asymmetry were used: the TF% (Huziwara, 1962), the AsK% (Arano, 1963) and the Syi (Greilhuber and Speta, 1976; Venora *et al.* 2002).

RESULTS

The physical distribution of the 45S and 5S rDNA were investigated by fluorescent *in situ* hybridization (FISH) (Figures 1 to 3) in two cytotypes of *Sprekelia formosissima*, a diploid ($2n=2x=60$) and a pentaploid ($2n=5x=150$). The diploid ($2n=2x=60$) cytotype showed

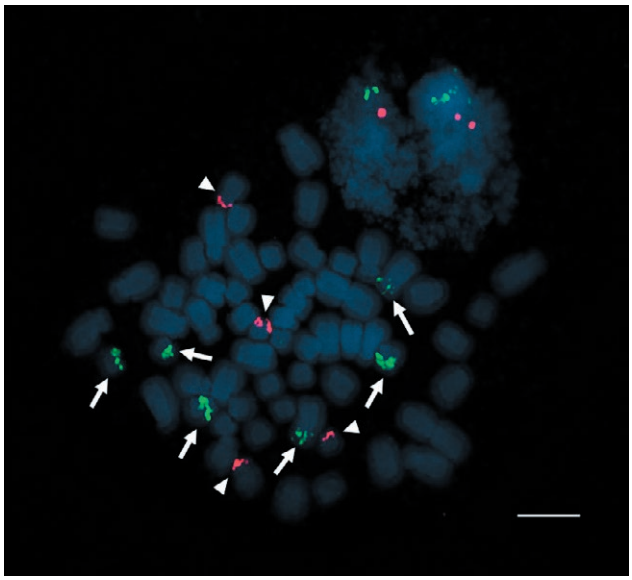

Figure 1. Fluorescent *in situ* hybridization of wheat 45S (red signals, arrowheads) and 5S rDNA (green signals, arrows) in a diploid *Sprekelia formosissima* ($2n=2x=60$) cytotype. The chromosomes were counterstained with DAPI. Bar=10µm.

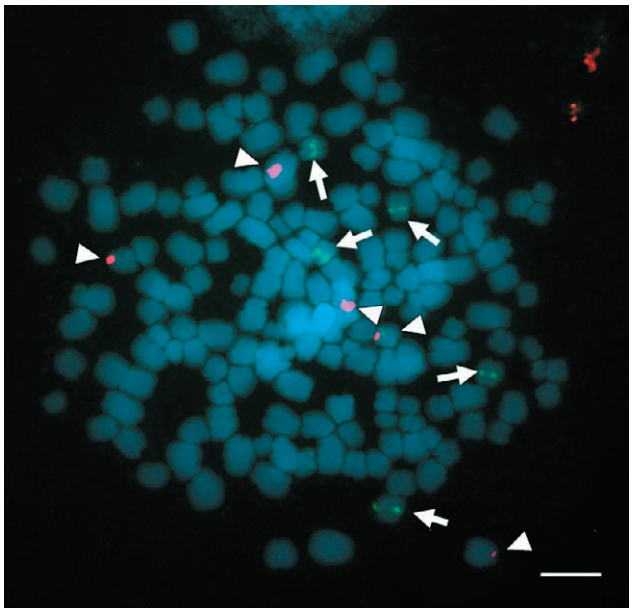

Figure 2. Fluorescent *in situ* hybridization of wheat 45S (red signals, arrowheads) and 5S rDNA (green signals, arrows) in a pentaploid *Sprekelia formosissima* (2*n*=5*x*=150) cytotype. The chromosomes were counterstained with DAPI. Bar=10μm.

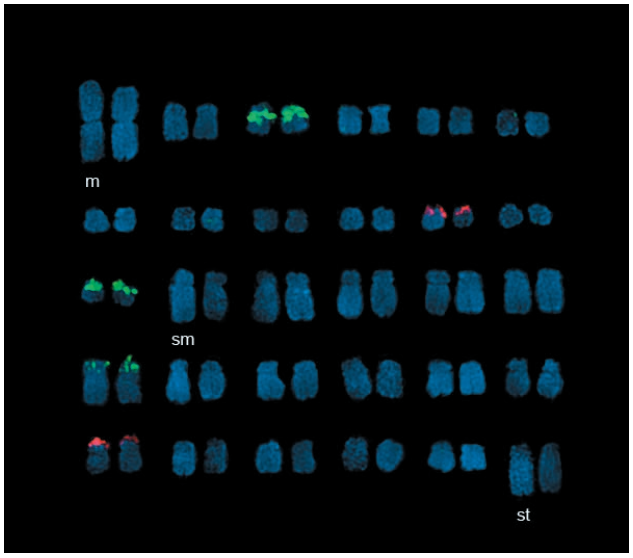

Figure 3. Karyogram of diploid *Sprekelia formosissima*. The wheat 45S rDNA hybridization *loci* (red signal) are present in chromosome pairs 11 and 25, while the wheat 5S rDNA *loci* (green signal) are present in chromosome pairs 3, 13 and 19.

four *loci* for the 45S rDNA in two chromosome pairs (11 and 25) in telomeric position. The 5S rDNA was present in six *loci* of three homologous chromosome pairs (3, 13 and 19) in subtelomeric and telomeric positions (Figures 1 and 3). Chromosome pair 1 was identified due to its unmistakable large size. The chromosomes of the pentaploid cytotypes (2*n*=5*x*=150) showed five *loci* for the 45S rDNA in telomeric position and five *loci* for the 5S rDNA in subtelomeric position (Figure 2). The hybridization *loci* for each probe was found always in different chromosomes. Thirty chromosome pairs (60 chromosomes in total) were identified in the diploid cytotype of *S. formosissima* and were arranged based on arm's ratio according to Levan *et al.* (1964) in the karyogram shown in Figure 3. The karyotypic formula was 13m + 16sm + 1 st with predominance of m and sm chromosomes. The longest per shortest chromosome ratio (L/S) ranges from 1.00 to 5.12. The karyotype symmetry/asymmetry index is TF % = 34.67, AsK % = 65.32 and Syi % = 54.81, concluding that it is an asymmetric karyotype, bimodal

Table 1. Chromosome parameters of *Sprekelia formosissima*

Chromosome pair no.	Total length (μm)	Long arm (L) (μm)	Short arm (S) (μm)	Arm ratio L/S	Centromeric position
1	10.42	5.40	5.02	1.08	M
2	5.02	3.10	1.92	1.61	M
3	3.98	2.29	1.69	1.36	M
4	3.86	2.22	1.64	1.35	M
5	3.60	1.96	1.64	1.20	M
6	3.41	1.95	1.46	1.34	M
7	3.26	1.71	1.55	1.10	M
8	3.24	1.89	1.35	1.40	M
9	3.19	1.81	1.38	1.31	M
10	3.14	1.89	1.25	1.51	M
11	3.06	1.55	1.51	1.03	M
12	2.87	1.57	1.30	1.21	M
13	2.61	1.36	1.25	1.09	M
14	6.84	4.77	2.07	2.30	Sm
15	6.46	4.70	1.76	2.67	Sm
16	6.15	4.25	1.90	2.24	Sm
17	6.08	4.46	1.62	2.75	Sm
18	6.04	4.3	1.74	2.47	Sm
19	5.50	4.12	1.38	2.99	Sm
20	5.43	4.07	1.36	2.99	Sm
21	5.22	3.77	1.45	2.60	Sm
22	5.16	3.63	1.53	2.37	Sm
23	5.14	3.63	1.51	2.40	Sm
24	5.04	3.32	1.72	1.93	Sm
25	4.91	3.19	1.72	1.85	Sm
26	4.61	3.15	1.46	2.16	Sm
27	4.31	2.92	1.39	2.10	Sm
28	4.16	2.68	1.48	1.81	Sm
29	3.68	2.50	1.18	2.12	Sm
30	6.84	5.41	1.43	3.78	St

with one distinctively large pair of chromosomes (10.42 µm) and a gradual decrease in the size of the other chromosome pairs, from the longest of 6.84 µm, to the shortest of 2.61 µm. (Table 1).

DISCUSSION

According to Roa and Guerra (2012), the most frequent *loci* number of 45S rDNA in angiosperms is two or four per diploid karyotype and they are generally located in terminal regions of the chromosomes, found more frequently in the short arms. In the cytotypes of *S. formosissima* analyzed in this work, both signals (45S and 5S rDNA) were detected in the short arms of the chromosomes. Even though the localization sites and the number of copies of the 5S and 45S *loci* present variation, it is probably not related to the ploidy level as has been suggested in some studies (Weiss-Schneeweiss and Schneeweiss 2013). This coincides with our results where four 45S rDNA sites and six 5S rDNA sites were detected in diploid plants, while five 45S rDNA sites and five 5S rDNA sites were detected in the pentaploid cytotype. There may be a different number of signals in species that have the same ploidy level, for example, García (2015) reported four 45S sites and nine 5S sites for diploid *Sprekelia howardii* Lehmiller, while in our work only four 45S sites were detected and six 5S sites for diploids analyzed. Differences in signal sizes were also detected even when they were in homologous chromosomes, which may be due to the existence of different copy numbers of the rDNA genes in each chromosome. There was a difference regarding the relation of the number of signals of rDNA in the cytotypes analyzed in this study; in the diploid cytotype there was less 45S signals with respect to 5S signals, while a similar number of rDNA signals were detected in the pentaploid cytotype. Based on the chromosome number of the diploid cytotype ($2n$=60) it can be considered as a polyploid due to its high chromosome number (Stebbins 1971), with this in mind, the lower 45S rDNA *loci* could be explained since these are generally more prone to experience rapid homogenization, silencing, and loss of *loci*, especially in polyploids (Clarkson *et al.* 2005; Kovařík *et al.* 2005; Weiss-Schneeweiss *et al.* 2008; Kotseruba *et al.* 2010). According the symmetry/asymmetry indexes used, the analyzes established that it is an asymmetric karyotype, however due to the predominance of metacentric and submetacentric chromosomes, it was impossible to differentiate them only by their size, for this reason the number of individual chromosomes identified was relatively low. In *S. formosissima* it was observed that the number of signals in the pentaploid cytotype does not correspond to a multiple of the number of signals present in the diploid cytotype, so it is concluded that at least for the wheat rDNA probes used in this work, the Ploidy level of *S. formosissima* is not related to the number of observed signals. The cytotype where the greatest number of chromosomes could be identified was the diploid *S. formosissima* ($2n=2x=60$), where two chromosomal pairs were identified with the 45S rDNA probe, three pairs with 5S rDNA probe and pair 1 due to its unmistakable large size (Figure 3), so a total of six chromosomal pairs could be identified.

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The role of oleuropein against nanocomposite toxicity in fruit fly: evidence for lifespan extension

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Abstract. The effect of zinc oxide/titanium dioxide nanocomposite the lifespan of the fruit fly *Drosophila melanogaster* and the protective role of oleuropein, a strong antioxidant, against the zinc oxide/titanium dioxide were investigated. Chemicals prepared in different concentrations (0.005, 0.1, 0.5, and 1 g/L for zinc oxide/titanium dioxide; 0.1 mmol/L for oleuropein) have been separately applied to female and male populations of *D. melanogaster* for the control and application groups. In both female and male populations, it has been observed that zinc oxide/titanium dioxide has decreased the lifespan and oleuropein has increased the lifespan according to the control group, depending on the concentration. These findings demonstrate the beneficial effect of oleuropein, suggested as a protective role in the prevention of zinc oxide/titanium dioxide induced developmental toxicity.

Keywords: *Drosophila melanogaster*, lifespan, oleuropein, toxicity, zinc oxide/titanium dioxide nanoparticles.

INTRODUCTION

Nanotechnology has a great potential for applications in many fields advancements in nanotechnology are increasing rapidly (Karahalil 2013). So, this technology is appearing as a new research field that is investigating the potential risks of nanoparticles (NPs) on human and environmental health (Landsiedel *et al.* 2009). In this respect, many reports have demonstrated various effects of NPs exposure on different animals, plants, and microorganisms; depending on their species, growth conditions, NPs type, and exposure concentrations. Also, the *in vitro* and *in vivo* studies using different experimental models indicate that nanoparticles may cause genotoxic effects that involve oxidative stress and inflammation (Kang *et al.* 2008; Xu *et al.* 2009; Petković *et al.* 2011). However, the mechanisms involved in NPs-induced toxicity have not been clearly explained and are poorly studied *in vivo*.

Metal NPs are the most widely used among nanoparticles. Titanium dioxide (TiO_2) and zinc oxide (ZnO) NPs are of special concern since they used in food products, plastics, paper, drugs, cosmetics, sunscreens, paints, and medical materials and are two of the fastest-growing product categories in the nanotechnology industry (Yeber *et al.* 2000). As a result of this widespread use, increased environmental release and a higher potential for human exposure will appear. The data from several *in vitro* studies demonstrate that TiO_2NPs cause various adverse effects at the cellular level, such as oxidative stress and DNA damage (Wang *et al.* 2007).

The aging process is generally described as the progressive decline of homeostatic maintenance functions and physiological fitness (Sohal *et al.* 2002). *Drosophila* or fruit fly is commonly used for studying in the aging model system because the genetic background, developmental processes, and some more aspects are well known. They are also highly similar to mammalians in terms of genetic structure (Çakır and Bozcuk 2000). The production of free radicals and other oxidants produced during aerobic respiration is the most reason for mechanistic explanations for aging links (Barja 2002). Oxidative stress is supposed to be an important mechanism underlying nanoparticle toxicity. Thus, it is believed that nanoparticle toxicity can be prevented by antioxidants (Nel *et al.* 2006; Mocan *et al.* 2010). From this point of view, the effects of zinc oxide/titanium dioxide (ZnO-TiO_2) nanocomposite on the lifespan of *D. melanogaster* and the protective role of oleuropein (OLE, Olive Leaf Extract), a strong antioxidant, on these effects.

MATERIALS AND METHODS

Experimental organism

Drosophila melanogaster (Diptera; Drosophilidae) *Oregon R* strain was reared in Standard *Drosophila* Medium (SDM) containing 15 g sucrose, 17 g cornmeal, 3 g agar-agar, and 9 g yeast in 360 mL distilled water within environmental chamber maintained at 25±1 °C and 40-60% relative humidity in darkness. Further addition of 1 mL propionic acid as a preservative/fungicide was done. The flies used in the experiments were at the same age (1-3 days) and the females were virgins.

Chemicals and solutions

Oleuropein (98% pure; St. Louis, MO, USA) and titanium dioxide (99% pure, anatase, Steinheim, Germany) were purchased from Sigma Aldrich. ZnO-TiO_2 (zinc oxide-titanium dioxide) nanoparticles were synthesized in the chemistry laboratory of Black Sea Technical University. ZnO was loaded into the TiO_2 photocatalyst with a 1% ratio. TiO_2 catalyst (10 g) and water (10 mL) were mixed to have a slurry. ZnO (0.3355 g) was added to the slurry and calcined at 400 °C for 6 h. After this process, it was cooled in a desiccator and stored in a closed dark bottle. For the stock solutions, $ZnOTiO_2$ NPs powder was dispersed in deionized water. Then, this solution was vortexed for 20 seconds, and sonicated for 30 min in an ultrasonic bath (Sonorex, Bandelin Electronic, Berlin, Germany) at a frequency of 60 kHz, to ensure uniform suspension of NPs. Finally, the nanoparticles concentrations were prepared by diluting the stock solution.

Characterization of $ZnOTiO_2$NPs

$ZnOTiO_2$NPs size distribution and morphology were represented by scanning electron microscope (SEM, (JEOL JSM 5600 LV, Tokyo, Japan) at the magnification of 100×. The hydrodynamic diameter was characterized by a master sizer (Malvern, Zetasizer ver. 7.02, Malvern Instruments Ltd, Worcestershire, UK) using the dynamic light scatter (DLS) technique.

Lifespan assay

The lifespan experiments were studied separately in female and male populations. For this purpose, about 100 individuals were collected from among the same aged (1-3 days) female and male flies which were not mated and obtained from the pupa. The gathered individuals were then put into the empty culture bottles and left hungry for 2 hours before $ZnOTiO_2$ and OLE applications. For the application, two layers of blotting papers were placed into each culture vial; $ZnOTiO_2$ and OLE in different concentrations (0.005, 0.1, 0.5, and 1 g/L for $ZnOTiO_2$, 0.1 mmol/L for OLE) were absorbed into these papers. Afterward, the flies were put into these application vials and were left for 2 hours. After 2 hours, the individuals (separately female and male flies) were placed into the culture vials containing only SDM as 25 × 25. The experiments for both the control and application groups were started at the same time. All the vials were kept in appropriate thermal cabins. During the experiments, food was replaced with fresh food twice a week. The number of individuals was controlled both at the beginning and the end of each application day and the dead individuals were counted. The application was carried out until the last individual died. The experiments were repeated three times.

Statistical analyses

The obtained data were analyzed with SPSS version 16.0 (Statistical Package for the Social Sciences Software, SPSS, Chicago, IL). The mean lifespan values of the control and application groups were subjected to Duncan's one-way range test ($p<0.05$).

RESULTS

Characterization of ZnOTiO$_2$NPs

The size of the nanoparticles is an important parameter that determines activity in biomedical applications. DLS is an analytical method that estimates the hydrodynamic diameter while SEM is used for the estimation of the actual diameter of nanoparticles. DLS studies revealed that the hydrodynamic diameter of ZnO-TiO$_2$NPs was 42.5±1.2 nm. The morphological characterization of ZnOTiO$_2$NPs was performed by SEM to visualize the actual particle size and the overall size distribution (Figure 1). SEM image indicates that the nanoparticles formed aggregates of different sizes and these aggregations have a porous structure.

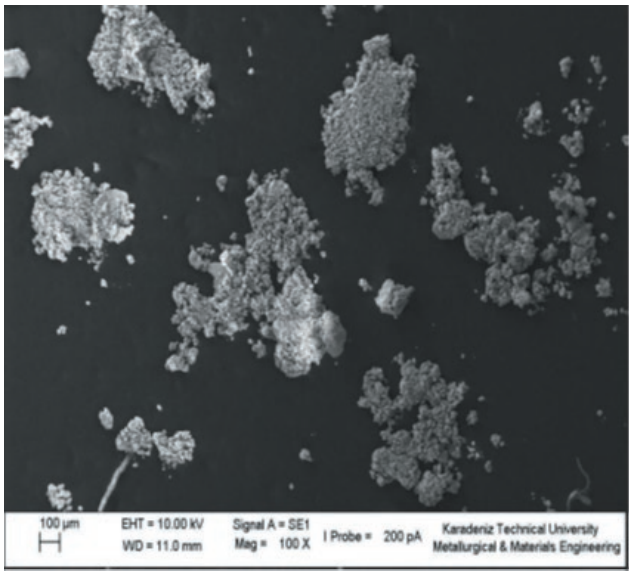

Figure 1. SEM images of ZnOTiO$_2$NPs in dry form.

Lifespan assay

In this study, it was observed that ZnOTiO$_2$, depending on the concentration, has decreased the lifespan of the male and female population according to the control group. It was also determined that OLE has increased the lifespan according to the control group. The maximum female lifespan of the control and application groups was observed for 78 days while the maximum male lifespan belonging to the control and application groups was 76 days, respectively. The difference between the control and application groups is not statistically significant ($p>0.05$) (Table 1).

In the female population applied with ZnOTiO$_2$, the maximum lifespan for the lowest concentration (0.005 g/L) was 70 days however for the highest concentration (1 g/L) the maximum lifespan was 54 days. Also, in the 0.1 and 0.5 g/L ZnOTiO$_2$ application groups, the female maximum lifespan was 68 and 62 days, respectively (Figure 2).

According to results, in the male population applied with ZnOTiO$_2$, the maximum lifespan for the lowest concentration (0.005 g/L) was 71 days however for the highest concentration (1 g/L) the maximum lifespan was 51 days. Also, in the 0.1 and 0.5 g/L ZnOTiO$_2$ applica-

Table 1. The longevity of male and female populations of *D. melanogaster*

Experimental group (No)	Female number	Maximum lifespan	Mean lifespan	Probability level	Male number	Maximum lifespan	Mean lifespan	Probability level
CONTROL - (1)	100	72	56.58±1.10		100	74	55.79±1.12	
OLE (0.1 mmol/L)- (2)	100	78	57.64±1.23		100	76	55.51±1.17	
ZnOTiO$_2$ 5 mg/L- (3)	100	70	49.48±1.45	1-2*	100	71	44.12±1.84	1-2* 3-4*
0.1 g/L- (4)	100	68	45.17±1.59	4-7*	100	66	41.03±1.85	3-7* 4-5*
0.5 g/L- (5)	100	62	35.41±1.29	5-6*	100	60	39.11±1.70	4-7*
1 g/L- (6)	100	54	34.19±1.65		100	51	35.50±1.45	5-6*
ZnOTiO$_2$ +OLE (1 g/L+0.1 mmol/L)- (7)	100	64	43.77±1.79		100	62	43.80±1.69	

*The mean difference is not significant at the 0.05 level.

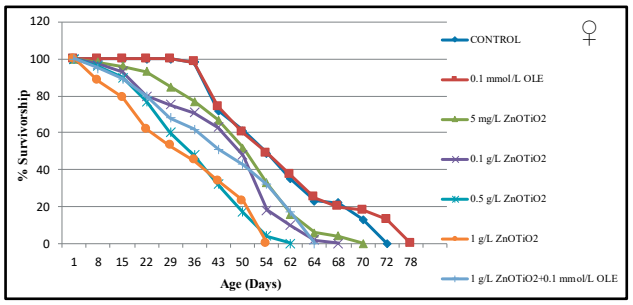

Figure 2. Exposure to $ZnOTiO_2$ and OLE in female adult *D. melanogaster* leads to lifespan alteration.

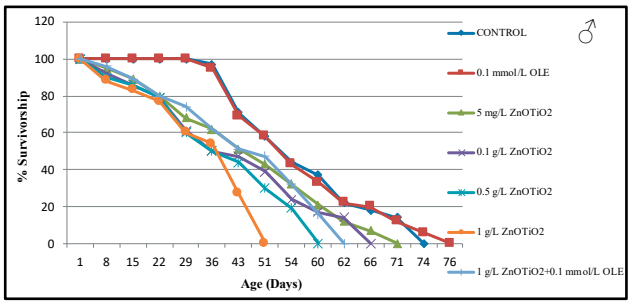

Figure 3. Exposure to $ZnOTiO_2$ and OLE in male adult *D. melanogaster* leads to lifespan alteration.

tion groups, the male maximum lifespan was 66 and 60 days, respectively (**Figure 3**). Also in every group, it is determined that female individuals live longer in comparison to male individuals (Table 1).

DISCUSSION

In recent years, studies about the toxic risks of NPs are increasing (Landsiedel *et al.* 2009; Yilmaz Öztürk 2019). First reports about the toxicity of some nanoparticles show that they can affect biological systems at the organ, tissue, cellular, subcellular, and protein levels (Braydich-Stolle *et al.* 2009; Khajavi *et al.* 2019). *In vivo* toxicity studies have demonstrated that inhalation of TiO_2 NPs causes pulmonary inflammation in rats and mice (Bermudez *et al.* 2004) and TiO_2 NPs induce DNA damage and genetic instability in mice (Trouiller *et al.* 2009). Reeves *et al.* (2008) showed oxidative stress-related effects, including inflammation, cytotoxicity, and genomic instability, either alone or in the presence of UVA irradiation, in mammalian studies. Also, zinc oxide nanoparticles have been reported to be cytotoxic and exhibited strong protein adsorption abilities (Horie *et al.* 2009). Toxicity of ZnO NPs on human bronchial epithelial cells was investigated and suggested that oxidative stress is a mechanism of toxicity (Heng *et al.* 2010). The production of free radicals has been supposed to be one of the primary mechanisms of NPs toxicity (Nel *et al.* 2006; Yang *et al.* 2009). It may result in oxidative stress, inflammation, and consequent damage to proteins, membranes, and DNA (Bhabra *et al.* 2009; Hu *et al.* 2009). Thus, in our study, we investigated the toxic effects of $ZnOTiO_2$ nanocomposite on the lifespan of *D. melanogaster* and the protective role of oleuropein (OLE), a strong antioxidant. Oleuropein is the major phenolic constituent of olive leaves (*Olea europaea*) and is also present in the fruit and oil (van Acker *et al.* 1998). Many studies demonstrated that OLE has an antiinflammatory activities (Carluccio *et al.* 2003), free-radical scavenging properties (Le Tutour and Guedon 1992; Manna *et al.* 1997) and inhibit the growth of different tumor cell types (Hamdi and Castellon 2005). In our preliminary study, it was determined that $ZnOTiO_2$NPs were relatively increased levels of total oxidant status (TOS) and was decreased level of total antioxidant capacity (TAC) compared to the control group. But, in a $ZnOTiO_2$+OLE group, it was observed that vice versa (Çolak *et al.* 2016).

CONCLUSION

These experiments provide evidence that $ZnOTiO_2$ nanocomposite can shorten the lifespan of *Drosophila* which is partially or completely prevented by oleuropein. This means oxidative stress is the major contributor to $ZnOTiO_2$ toxicity. As a result, although the emerging technologies and their opportunities are desirable to be implemented into daily life, before their applications, regulations should be defined to protect human health and the environment.

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Pollen and floral micromorphological Studies of the genus *Cotoneaster* Medik. (Rosaceae) and its systematic importance

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Abstract. The micromorphology of petal and pollen grain of 16 species of the genus *Cotoneaster* Medik., belonging to two subgenera (*Cotoneaster* and Chaenopetalum) has been studied by light and scanning electron microscopy. We used different multivariate statistical methods to reveal the species relationships. Results showed that in comparison to most genera of the family Rosaceae, both tri- and tetracolporate pollen grains are observed in one specimen. Palynological observation revealed that shape of tricolporate pollen in most species is prolate-spheroidal, but also subprolate and prolate pollen grains can be recognized. In the other hand, tetracolporate pollen is quadrangular. The main ornamentation type was mainly striate which in turn can be subdivided to several categories; however, psilate one is also recognized also (*C. persicus*). The results revealed that pollen traits are probably effective in separating the sections and using these traits for placing a species in a particular section is probably helpful. Apomixis is one of the reasons for not changing the ornamentation of the both surface of the petals and their similarity to each other in different species. Totally, the studied micromorphological characters of petal cannot be used as diagnostic tools for *Cotoneaster* in Iran.

Keywords: Cotoneaster, Iran, pollen, petal, Rosaceae, SEM.

INTRODUCTION

The genus Cotoneaster Medik. which is mostly a shrubby member of the subtribe Pyrinae, tribe Pyreae, subfamily Spiraeoideae, family Rosaceae (Campbell *et al.* 2007). *Cotoneaster* consists of about 90 species widespread in temperate Asia (except Japan), Europe and North Africa (Yü and Lu 1974; Lu and Brach 2003), although other authors consider the number of species 260 (Mabberley 2008) to 400 (Fryer and Hylmö 2009). The center of diversity for the genus is in Tibet and the Himalayas, though species are native across Asia, North Africa, and Europe (Bartish *et al.* 2001; Dickore and Kasperek 2010; Fryer and Hylmö 2009). In Iran, 19 species of the genus are mainly dis-

tributed in Alborz Mts., elevations in NW (Azerbaijan province) and NE (Khorasan province) (Raei Niaki *et al.* 2009). Among these species, the *C. assadii*, *C. esfandiarii* and *C. persicus* are endemic to Iran (Riedl 1969; Khatamsaz 1985; Khatamsaz 1992).

The petal traits or the number of flowers in the cyme is the main characters used to determine interspecific relationships in *Cotoneaster* (Koehne 1893, Yu 1963). In some families, petal morphology is one of the most important diagnostic characters (Sharma *et al.* 2005, Campbell *et al.* 2007, Akcin 2009, Arianmanesh *et al.* 2016). The patterns of petal epidermis in angiosperms particularly Rosaceae family have important characters for identification of close species (Christensen and Hansen 1998). Several researchers have focused on petal micromorphology of different genera of Rosaceae (Tahir *et al.* 2010, Sharifnia and Behzadi Shakib 2012, Omer *et al.* 2017).

Regarding pollen morphology, it has been proved to be beneficial in systematic of the family Rosaceae (Hebda and Chinnappa 1990); however some others deny such an application (Moore *et al.* 1991) which is caused by easy hybridization among several species and even genera of the family. Regarding *Cotoneaster*, some a few studies (Kumar and Panigrahi 1995; Hsieh and Huang 1997; Perveen and Qaiser 2014) have reported some common features of pollen such as size, aperture number, exine thickness and ornamentation of surface. According to these studies, ornamentation of pollen surface including striate, sub-psilate and regulate ones is the most important feature in separating species. However, generally they emphasized these characters only play a little role in separating a few species and pollen morphology is not a useful tool in classification of the genus.

A comprehensive study on morphological and micro-morphological characters in *Cotoneaster* is almost lacking, moreover, the potential application of these characters in taxonomy of the genus has not been illustrated yet. Therefore, the objectives of the present study were 1) to provide detailed morphological and micromorphological information on petal and pollen morphology of *Cotoneaster*, and 2) to evaluate application of these characters to find out the inter species relationships and delimit the species taxonomically.

MATERIAL AND METHODS

Pollen sampling

Totally 42 populations were collected and studied from 16 taxa of *Cotoneaster* from different habitats in Iran for study the pollen features (Table 1). 5-8 individuals of each location were studied and examined for 2 qualitative and 13 quantitative features (Table 2 and 3). Voucher specimens were deposited in TUH and FUMH (Table 1). Pollen obtained from flower buds at anthesis were prepared for light microscope (LM) using methods described by Harley (1992) with some modifications, mounted in glycerol jelly on glass slides and sealed. For LM measurements, at least 20–25 pollen grains were measured by Nikon light microscope model 200 M with aid of a × 100 eyepiece. For scanning electron microscopy (SEM) examinations, pollen grains were not acetolysed according to the method of Erdtman (1960). The pollen were suspended in a drop of water for a while, and then directly transferred to a metallic stub by a fine pipette, and double sided cello tape were used and then the pollen were sputtered in chamber coated with gold (Sputter Coater BALTEC, SCDOOS). Coating with gold by the physical vapor deposition method (PVD) was restricted to 100 Å. The SEM examination was carried out on a TESCAN microscope. For detailed examination of sculpturing, the classification presented in Ueda and Tomita (1989) was used. For estimation of pollen fertility, the pollen from fresh collected herbarium materials were stained by acetocarmine glycerin jelly, as described by Radford *et al.* (1974).

Petal sampling

In the current study, the micromorphological characteristics of petals of 16 species belonging to two subgenera of the *Cotoneaster* (*Cotoneaster* and *Chaenopetalum*) were studied for the first time. The collected specimens were deposited in the herbarium of Tehran University (TUH), Ferdowsi University of Mashhad Herbarium (FUMH) (Table 1). The voucher specimens are listed in Table 1. 122 specimens from 42 different populations of *Cotoneaster* taxa were collected from their natural habitats in Iran. Five micromorphological characters were examined; among the five characters, four were qualitative and one character was quantitative (Table 4).

The materials for SEM observation were directly mounted on stubs without any treatment, and sputter coated with gold-palladium. Petals and sepals of fully opened flowers were investigated using a HITACHI model SU 3500 electron microscope at 15 kV accelerating voltage; After a number of specimens had been compared under SEM. Both petal surfaces were examined. The epidermis of the petals was classified based on cell ornamentation, shape of cell (the primary sculpture), visibility of the boundary between cells using the terminology of Barthlott (1981) and Kay *et al.* (1981).

Table 1. List of the investigated taxa including origin of voucher specimens.

Taxon	Voucher information
C.* subgen. *Cotoneaster	
C. melanocarpus (Ledeb.) Lodd., G. Lodd. & W. Lodd. ex M. Roem.	Mazandaran: Firoozkooh road, 30 km after Veresk village to Tehran, after Dogol station. Raei Niaki & Mahdigholi. 46888-TUH
C.* subgen. *Chaenopetalum	
C. multiflorus Bunge.	Kurdistan: Nushoor olia village; Attar, Raei Niaki & Maroofi, 46870-TUH
C. suavis Pojark.	Khorasan: Gifan, Misino mountain, 20790-FUMH
C. hissaricus Pojark.	Azerbayjan: after Peygham village to Kaleybar; Attar, Zamani & Raei Niaki, 37261-TUH.
C. morulus Pojark.	Azerbayjan: Orumiyeh, Marmishu lake, Attar & Zamani, 40614-TUH
C. tytthocarpus Pojark.	Mazandaran: Siah-bishe, 7 km after Pole-Zanguleh to Chalus. Raei Niaki & Mahdigholi, 46887-TUH
C. luristanicus G. Klotz	Luristan: Aleshtar. Ghahraman, Attar & Ghaffari. 21658-TUH
C. turcomanicus Pojark.	Mazandaran: Firoozkooh Road, 30 Km after Veresk village to Tehran; Raei Niaki & Mahdigholi. 46890-TUH
C. nummularioides Pojark.	Mazandaran: Chalus road, between Reyzamin and Asara village; Attar, Zamani & Raei Niaki; 37203-TUH
C. kotschyi (C.K.Schneid.) G.Klotz	Mazandaran: Haraz Road, Yush village. Raei Niaki & Mahdigholi, 46897-TUH
C. assadii khat.	Mazandaran: Siah-bishe, Gachsar village; Raei Niaki & Mahdigholi, 46898-TUH
C. nummularius Fisch. & C.A.Mey.	Mazandaran: Firoozkooh Road, Seleben Village; Raei Niaki & Mahdigholi, 46901-TUH
C. ovatus Pojark.	Mazandaran: Firoozkooh Road, Seleben Village; Raei Niaki & Mahdigholi, 46892-TUH
C. esfandiarii khat.	Mazandaran: Firoozkooh, Arjmand village, Attar & Raei Niaki, 46886-TUH
C. discolor Pojark.	Mazandaran: Firoozkooh Road, 30 km after Veresk village to Tehran, Raei Niaki & Mahdigholi, 46889-TUH
C. persicus Pojark.	Khorasan: Pivehjan village, 23394-FUMH

Data analysis

The characters of pollen grains of the studied species are summarized in Tables 2 and 3. Multi-state qualitative characters converted into presence-absence descriptions. 13 pollen grain quantitative data were noted and treated statistically to determine average values for each species. PCA analysis were performed to check the similarity and dissimilarity between different taxa of the tribe, after linear standardization by range of each variable of the original data set. Principal Components Analysis (PCA) was performed to check the dissimilarity between the studied species based on palynological features useful for the delimitation of the species. To calculated Euclidean and taxonomic distance between different species of the genus, PCA ordination plot was performed (Podani 2000). PAST version 2.17 (Hammer *et al.* 2012) was used for multivariate statistical analyses of morphological data.

RESULTS

In the present investigation different micro morphological characters of the petal and pollen grain of *Cotoneaster* have been studied in detail. The investigated specimens are given in Table 1. The petal and pollen morphological characters are summarized in Table 2, 3 and 4.

Pollen morphology

The most important characters are given in table 2 and 3. Selected micrographs are presented in (Figures 1, 2, 3). Pollen grains are shed as monad, medium-sized (P= 29.26–35.13). One important and interesting feature observed frequently in most of the studied species, is the presence of tri- and tetracolporate pollen grains in the same specimen (e.g. Figures 1. e, o; Figures 2. e, k, q), while most species of family Rosaceae consist of tricolporate pollen. Percentage of this character is variable in different species, so that tetracolporate pollen in some species such as *C. melanocarpus* Fisch. ex A.Blytt , *C. kotschyi* (C.K.Schneid.) G.Klotz and *C. multiflorus* Bonge. is frequent, while the percentage of tricolporate pollen in some others such as *C. tytthocarpus* Pojrk. and *C. morulus* Pojark. constitute the major percentage of pollen grains. Regarding symmetry, both tricolporate and tetracolporate pollen grains are isopolar (e.g. Figures 1. i, k, m; Figures 2. c, e, f, g, m) and heteropolar (e.g.Figures 1. c, e, q). In the case of the shape of tri-

Table 2. Evaluated characters of pollen grains in *Cotoneaster* species studied (values M ± SD μm). M- Mean value; SD- Standard deviation. Abbreviations: P, polar axis length; E, equatorial axis length; P/E, proportion of polar axis to equatorial axis length; M, Mesocolpium length; A, Apocolpium length; C, Colpus length; E, Exine thickness

Taxa	P (μm)	E (μm)	P/E(μm)	M (μm)	A (μm)	C (μm)	E (μm)
C. Subgen.*Cotoneaster*							
C. Sect.*Cotoneaster*							
C. Ser. *Cotoneaster*							
C. integerrimus	31.0(33.73±2.12)39.0	23.0(28.20±3.76)35.0	1.3	12.00(18.53±4.61)25.00	5.00(6.60±1.40)9.00	27.00(29.47±2.26)35.00	0.70(0.97±0.12)1.10
C. Ser. *Melanocarpi*							
C. melanocarpaus	32.0(35.00±1.93)39.0	23.0(27.47±3.46)34.0	1.3	13.00(16.93±2.43)23.00	5.00(6.75±1.04)8.00	28.00(30.07±1.62)33.00	0.70(1.69±0.42)2.00
C. Subgen.*Chaenopetalum*							
C. Sect.*Chaenopetalum*							
C. Ser.*Racemiflori*							
C. persicus	29.00(33.40±2.85)39.00	18.00(25.20±4.77)35.00	1.37	11.00(17.93±5.19)30.00	4.00(5.87±1.12)8.00	25.00(29.47±2.39)34.00	1.00(1.71±0.32)2.00
C. discolor	30.00(31.10±1.28)34.00	25.00(28.20±2.73)33.00	1.11	17.00(21.73±4.32)30.00	5.00(6.33±1.18)8.00	22.00(22.47±1.99)30.00	0.70(0.97±0.12)1.10
C. assadii	30.00(32.43±2.35)35.00	23.00(26.00±3.67)33.00	1.25	16.00(20.53±5.73)25.00	4.00(6.12±1.32)9.00	23.00(26.05±1.25)28.00	0.85(1.34±0.56)1.30
C. nummularius	31.00(33.73±2.12)39.00	23.00(31.00±3.82)40.00	1.1	15.00(22.67±5.33)33.00	4.00(7.47±1.46)10.00	28.00(30.13±1.96)35.00	0.70(1.30±0.43)2.00
C. esfandiarii	28.00(30.93±2.31)35.00	23.00(28.20±3.76)35.00	1.11	16.00(21.93±4.88)32.00	4.00(9.27±1.44)8.00	23.00(26.00±2.03)30.00	1.00(1.79±0.45)2.60
C. ovatus	29.00(33.47±1.76)36.00	25.00(30.87±3.87)36.00	1.1	11.00(20.60±4.92)28.00	4.00(6.07±1.39)9.00	26.00(29.87±2.53)36.00	0.80(1.39±0.48)2.00
C. Ser.*Hissarici*							
C. hissaricus	30.00(33.80±2.01)38.00	21.00(27.27±3.55)34.00	1.26	12.00(18.53±4.61)25.00	5.00(6.93±1.03)9.00	27.00(29.47±2.26)35.00	0.90(1.09±0.20)1.70
C. turcomanicus	31.00(35.13±3.11)43.00	21.00(33.20±5.72)40.00	1.1	10.00(25.80±8.21)35.00	5.00(7.47±1.19)9.00	23.00(30.20±4.34)40.00	0.80(1.38±0.46)2.50
C. morulus	25.00(30.20±2.98)37.00	13.00(21.20±3.51)26.00	1.47	7.00(13.47±3.58)20.00	5.00(6.60±1.40)9.00	21.00(26.13±3.02)33.00	1.00(1.26±0.20)1.70
C. tytthocarpus	25.00(29.26±3.01)35.00	18.00(22.93±3.79)32.00	1.29	9.00(13.33±2.77)20.00	5.00(6.25±2.50)10.00	20.00(25.33±3.11)30.00	0.90(1.44±0.46)2.10
C. luristanicus	31.00(33.53±2.20)37.00	27.00(33.40±3.16)38.00	1.01	17.00(26.20±5.68)37.00	5.00(6.97±1.26)10.00	25.00(28.80±2.73)33.00	1.20(1.75±0.33)2.50
C. kotschyi	30.00(33.20±2.40)38.00	21.00(24.27±3.06)32.00	1.39	11.00(16.13±4.75)30.00	4.00(5.10±0.96)7.00	26.00(29.53±2.10)34.00	0.80(1.23±0.33)1.90
C. nummularioides	25.00(31.27±2.68)37.00	21.00(23.53±2.03)28.00	1.34	12.00(15.80±2.24)20.00	4.00(6.53±1.36)9.00	21.00(27.53±2.88)33.00	1.00(1.40±0.38)2.00
C. Sect.*Multiflori*							
C. Ser. *Multiflori*							
C. multiflorus	30.00(34.78±2.82)37.00	20.00(26.89±5.04)36.00	1.32	8.00(14.56±4.19)21.00	5.00(7.21±1.25)9.00	25.00(29.67±3.08)34.00	1.50(1.76±0.22)2.00

Table 3. Continue evaluated characters of pollen grains in *Cotoneaster* species studied (values M ± SD μm). M- Mean value; SD- Standard deviation. Abbreviations: C/P, proportion of colpus to polar axis length; S, Shape of pollen; Sc, Sculpturing of pollen; FP, Fertility percentage

Taxa	C/P	S	Sc	FP	pore number	ridge width	inter ridge width	pore width
C. Subgen.*Cotoneaster*								
C. Sect.*Cotoneaster*								
C. Ser. *Cotoneaster*								
C. integerrimus	0.83	subprolate	TypeII-A	96%	2.00(8.87±3.00)13.00	0.19(0.22±0.03)0.28	0.04(0.08±0.02)0.14	0.05(0.14±0.05)0.22
C. Ser. *Melanocarpi*								
C. melanocarpaus	0.85	subprolate	TypeII-A	95%	7.00(10.33±2.47)16.00	0.18(0.31±0.08)0.47	0.05(0.14±0.07)0.37	0.05(0.21±0.09)0.38
C. Subgen.*Chaenopetalum*								
C. Sect.*Chaenopetalum*								
C. Ser.*Racemiflori*								
C. persicus	0.88	subprolate	Type VI	85%	4.00(6.11±1.80)9.00	0.08(0.18±0.05)0.24	0.07(0.13±0.04)0.22	0.07(0.16±0.03)0.26
C. discolor	0.72	prolate-spheroidal	Type V	97%	1.00(4.47±2.36)9.00	0.12(0.21±0.05)0.29	0.10(0.22±0.07)0.33	0.08(0.16±0.07)0.29
C. assadii	0.82	subprolate	Type III	92%	7.00(9.47±2.10)15.00	0.20(0.26±0.04)0.35	0.06(0.08±0.02)0.11	0.07(0.16±0.04)0.26
C. nummularius	0.89	prolate-spheroidal	Type I	98%	2.00(6.93±2.41)10.00	0.09(0.18±0.04)0.25	0.06(0.13±0.04)0.23	0.06(0.14±0.05)0.24
C. esfandiarii	0.84	prolate-spheroidal	Type I	98%	4.00(6.13±1.82)9.00	0.08(0.15±0.04)0.24	0.07(0.13±0.04)0.22	0.13(0.18±0.03)0.25
C. ovatus	0.89	prolate-spheroidal	Type III	98%	7.00(12.53±3.52)17.00	0.12(0.17±0.03)0.24	0.05(0.08±0.02)0.12	0.07(0.10±0.03)0.17
C. Ser.*Hissarici*								
C. hissaricus	0.87	subprolate	Type I	96%	6.00(8.27±2.41)14.00	0.10(0.16±0.04)0.22	0.07(0.16±0.07)0.30	0.09(0.19±0.06)0.34
C. turcomanicus	0.85	prolate-spheroidal	Type II-A	99%	2.00(5.93±2.29)9.00	0.17(0.27±0.07)0.45	0.13(0.20±0.04)0.29	0.18(0.23±0.03)0.30
C. morulus	0.86	prolate	Type II-B	96%	9.00(12.40±3.16)21.00	0.15(0.22±0.05)0.33	0.07(0.14±0.04)0.20	0.08(0.15±0.07)0.33
C. tytthocarpus	0.86	subprolate	Type I	90%	0.00(5.73±4.12)14.00	0.18(0.29±0.06)0.38	0.06(0.10±0.02)0.13	0.08(0.16±0.08)0.39
C. luristanicus	0.85	prolate-spheroidal	Type III & IV	99%	5.00(7.73±1.65)10.00	0.13(0.21±0.05)0.29	0.07(0.14±0.05)0.23	0.10(0.31±0.14)0.59
C. kotschyi	0.88	prolate	Type V	95%	3.00(5.07±1.12)7.00	0.13(0.18±0.03)0.26	0.09(0.14±0.03)0.21	0.08(0.19±0.06)0.30
C. nummularioides	0.88	prolate	Type V	88%	0.00(12.20±5.13)20.00	0.14(0.24±0.05)0.34	0.08(0.11±0.02)0.15	0.08(0.12±0.04)0.24
C. Sect.*Multiflori*								
C. Ser. *Multiflori*								
C. multiflorus	0.85	subprolate	Type I & IV	96%	0.00(4.13±3.01)9.00	0.14(0.25±0.05)0.35	0.08(0.12±0.02)0.16	0.07(0.17±0.07)0.34

Table 4. Distribution and coding of main petal characteristics in studied species.

Taxon	Number of conical projections in 50 μm²	Distinct or not distinct Boundaries between cells	Closely or not closely conical projections	Folding or not folding of top of conical projections	Oriented or not oriented of conical projections
C. subgen. *Cotoneaster*					
C. melanocarpus	12	_	+	+	_
C. subgen.*Chaenopetalum*					
C. multiflorus	9	+	_	_	+
C. suavis	9	_	+	+	_
C. hissaricus	13	+	_	+	+
C. morulus	12	+	_	+	+
C. tytthocarpus	19	_	+	_	_
C. luristanicus	16	_	+	+	_
C. turcomanicus	11	_	+	+	+
C. nummularioides	12	+	_	_	+
C. kotschyi	17	_	+	_	_
C. assadii	17	+	_	+	_
C. nummularius	25	_	+	_	_
C. ovatus	8	+	_	+	_
C. esfandiarii	18	_	+	+	+
C. discolor	17	+	+	_	+
C. persicus	13	+	_	_	_

colporate pollen in equatorial view, prolate- spheroidal (e.g. Figures 2. m), subprolate (e.g. Figure 2. i) and prolate (Figure 1. i) shapes (column S in Table 3) are recognized, while in polar view triangular (e.g. Figures 1.a, q) and trilobate (e.g Figures 1. h, Figures 2. a, k) shapes can be recognized. In the other hand, tetracolporate pollen are quadrangular (e.g. Figures 1. c, e, Figures 2. e, q). Shape of apex varies from obtuse (e.g. Figures 1. c, g, m, Figure 2. e) to truncate (e.g. Figure 1. E; Figures 2. g, i). Colpi which occupy 72% in *C. discolor* Pojark. to 89% in *C. nummularius* Fisch. and *C. ovatus* Pojark. of length of the polar axis, are arranged meridionally (e.g. Figure 1. G; Figure 2. e) or parallel (e.g. Figures 1 i, k; Figures 2. g, i). Endopores which are located in the middle of ectocolpi, consist of distinct (e.g. Figures 1. c, e, g) or indistinct (e.g. Figures 2. c, g, i) projections. The mean of polar axis length (column P in Table 2) varies from 29.26 μm in *C. tytthocarpus* to 35.13 μm in *C. turcomanicus* Pojark. while the mean of equatorial axis length (column E in Table 2) varies from 21.20 μm in *C. morulus* to 33.40 μm in *C. luristanicus* G. Klotz. The mean of mesocolpium axis length (column M in Table 2) varies from 13.33 μm in *C. tytthocarpus* to 26.20 μm in *C. luristanicus*. Regarding apocolpium axis length (column A in Table 2), range is from 5.10 μm in *C. kotschyi* to 9.27 μm in *C. esfandiarii*. The mean of colpus length (column C in Table 2) varies from 22.47 μm in *C. discolor* to 30.20 μm in *C. turcomanicus*. The thickness of exine (column E in Table 2) which is clearly composed of two layers (ectexine and endexine) varies from 0.97 μm in *C. discolor* to 1.79 μm in *C. esfandiarii* Khat. Results of fertility test showed that most species have high percentage of fertility so that this character (column FP in Table 3) ranges from 85% in *C. persicus* to 99% in *C. turcomanicus*. With regard to sculpturing, the prominent ornamentation is striate (e.g. Figures 1. d, f, l, p; Figure 2. h); however some others such as psilate (Figures 2. o, p) can be recognized. Also the sterile pollen grains have deformed shape (Figure 3. q).

As illustrated above, main feature of several species (i.e. sculpturing) is very homogenous in different species. But type of sculpturing, number of perforation and the perforation size is different in same species. For example, series *Hissarici* members represent a rather uniform group but different types of sculpturing are observed in these species. On the basis of this character, *C. persicus* is separated from other species with its psilate sculpturing (Figures 2. o, p). This species is closely related to *C. discolor*, but differs from it by subglabrous upper leaf surface (very sparsely pilose – strigose in *C. persicus*), red vein and petiole (green in *C. persicus*).

According to exine sculpturing pattern, two main types (striate) and non-striate (psilate) were recognized in the *Cotonoster*. Most of the specimens belong to types striate.

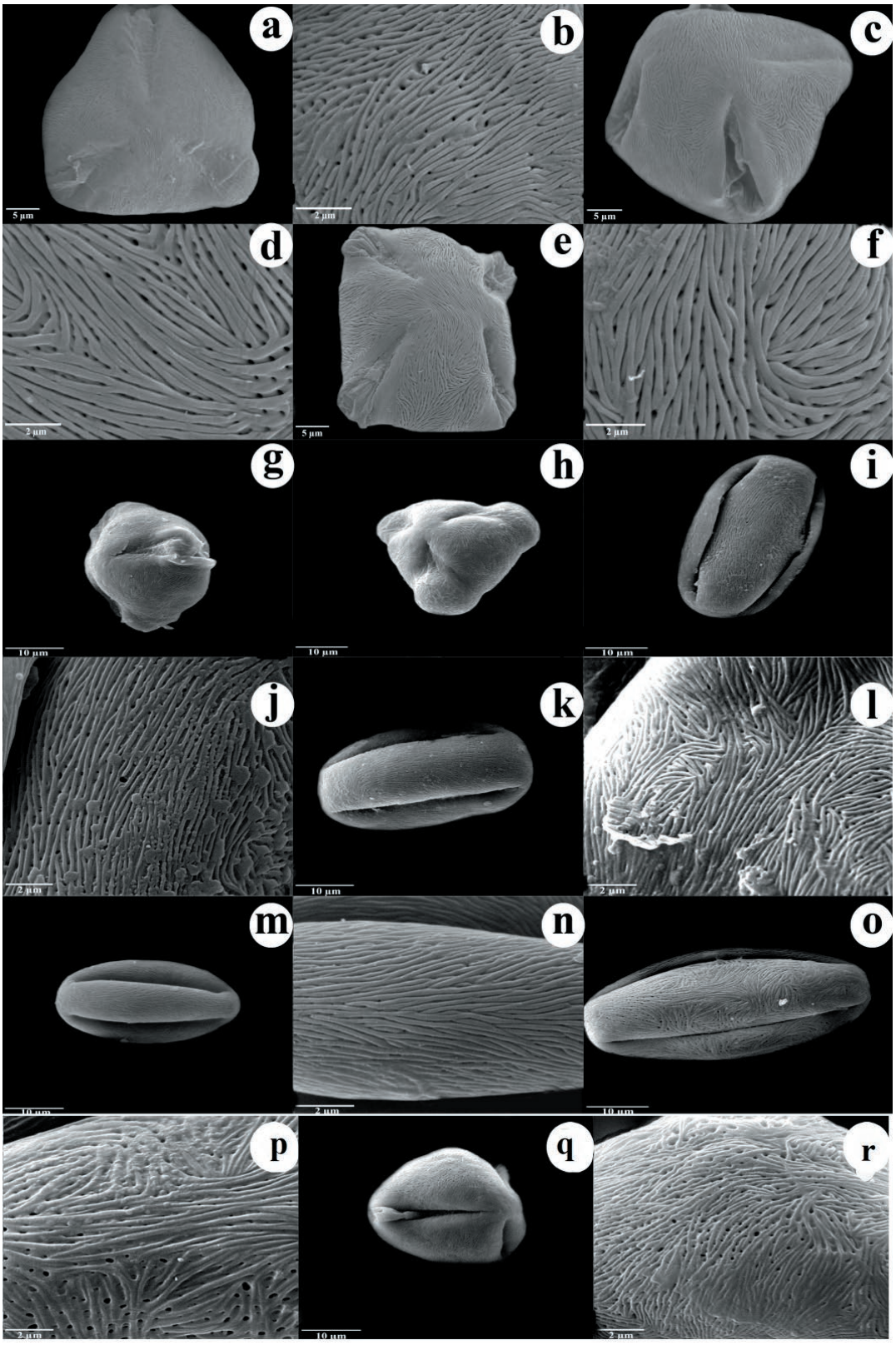

Figure 1. SEM micrographs of pollen grains of *C. integerimus* (a-b), *C. melanocarpus* (c-d), *C. turcomanicus* (e-f), *C. morulus* (g-j), *C. ovatus* (k-l), *C. assadii* (m-n), *C. luristanicus* (o-p), *C. nummularioides* (q-r).

Type (I): striate

This type is recognized by distribution of lira throughout pollen surface. This type is subdivided according to the pattern of perforation between lira.

Subtype I

Striate pollen, which has clear fingerprint-like ridges with few small perforations and with long intervals of ridges. This subtype is observed in these species: *C. nummularius*, *C. esfandiarii*, *C. hissaricus* Pojark., *C. tytthocarpus* and *C. multiflorus* (Figures 2 f, h, j, l, n).

The first two species surely belong to the subgenus *Chaenopetalum*, section *Chaenopetalum*, series *Racemiflori* (classification according Fryer and Hylmo, 2009). They have semi-dense inflorecensc and red fruit (except *C. esfandiarii*) and the two latter species are members of the *Hissarici* series that they have lax inflorecens and black fruit. This type also is observed in the last species; *C. multiflorus* from subgenus *Chaenopetalum*, section *Multiflori*, series *Multiflori* with open inflorescence and lower surface of leaves scarcely hairs but pollen of this species has very large perforation similar to that is seen in subtype IV.

Subtype type II (A-B)

This subtype differs from subtype I by having prominent perforations between ridges. This type is subdivided according to interval of ridges; subtype II-A and type subtype II- B with short and long intervals, respectively. Subtype II-A is observed in *C. integerrimus* Medik., *C. melanocarpus* and *C. turcomanicus* (Figures 1. b, d, f). Subtype II-B is seen in *C. morulus* (Figure 1. j)

Subtype III

This subtype differs from subtype II, subtype III has short ridges (0.15 to 0.30 μm) and can be seen in *C. assadii* Khat., *C. ovatus* and *C. luristanicus* (Figures 1. l, n, p). The first two species are members of the series *Racemiflori*, the latter species is the member of the *Hissarici* series.

Subtype IV

This subtype is diagnosed by having very large perforations.

Subtype V

This subtype is recognized by having obscure ridges due to very moderate slope of ridge. This subtype is seen in *C. discolor*, *C. kotschyi* and *C. nummularioides* Pojark. (Figures 2. b, d; Figure 1. r). First species belongs to the series *Racemiflori* and the latter two species are in *Hissarici* series.

Type (II): Psilate

This type is diagnosed by having no ridge on the pollen surface. This type is seen in *C. persicus* (Figures 2. o-r). This species is a member of the series *Racemiflori* and lacks any perforation on the surface.

Petal morphology

The micromorphological characters of petals of 16 species belonging to two subgenera of the *Cotoneaster* were studied. Also, according to previous studies on petals of other genera of Rosaceae, the ornamentations of the adaxial surface and the lower surface of the petals are described.

Adaxial surface of petals:

On the adaxial surface of all petals conical (finger-like or tubercle) shape projection are observed.

C. melanocarpus: The epidermal cells of the petal surface are loosely packed with distinct outline. This species exhibits irregular folds and rugose tuberculate pattern. The surface of each cell exhibits striate to rugose pattern. The ruga and striae are condensed and forming ruminate pattern on the tubercle of folds (Figure 4. a).

C. multiflorus: The petal surface cells are distinct and loosely packed with distinct cell walls. The margin of cells is smooth. The central part of the cells is raised into small regular finger-like projections. A tubercle is formed in the middle of the finger-like projection with ruminate patterns. The surface of each cell exhibiting striate to rugose pattern (Figure 4. b).

*C. suavis***:** Adaxial surface has loosely packed cells with prominent cell boundaries and more or less thick folds, forming tubercle in the middle of the folds. The surface as a whole is striate to rugose but at the tubercle becomes ruminate (Figure 4. c).

C. hissaricus: Petal surface of this species exhibits closely packed epidermal cells. The cell surfaces are raised into broad finger-like projections or tubercles. The surface as a whole shows striate pattern which is paral-

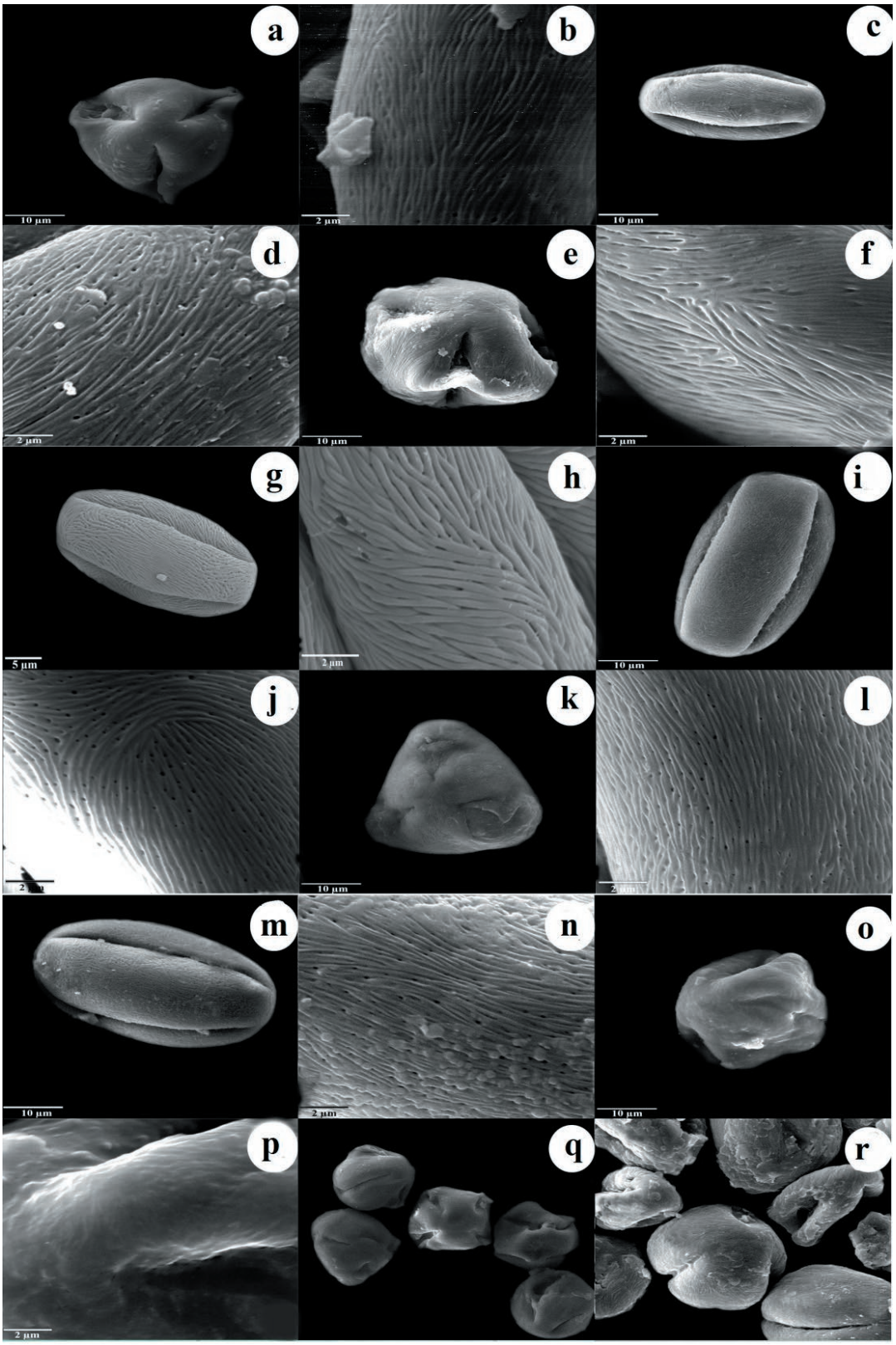

Figure 2. SEM micrographs of pollen grains of *C. kotschyi* (a-b), *C. discolor* (c-d), *C. multiflorus* (e-f), *C. tytthocarpus* (g-h), *C. hissaricus* (i-j), *C. nummularius* (k-l), *C. esfandiarii* (m-n), *C. persicus* (o-p), *multiflorus* (q), *C. discolor* (r).

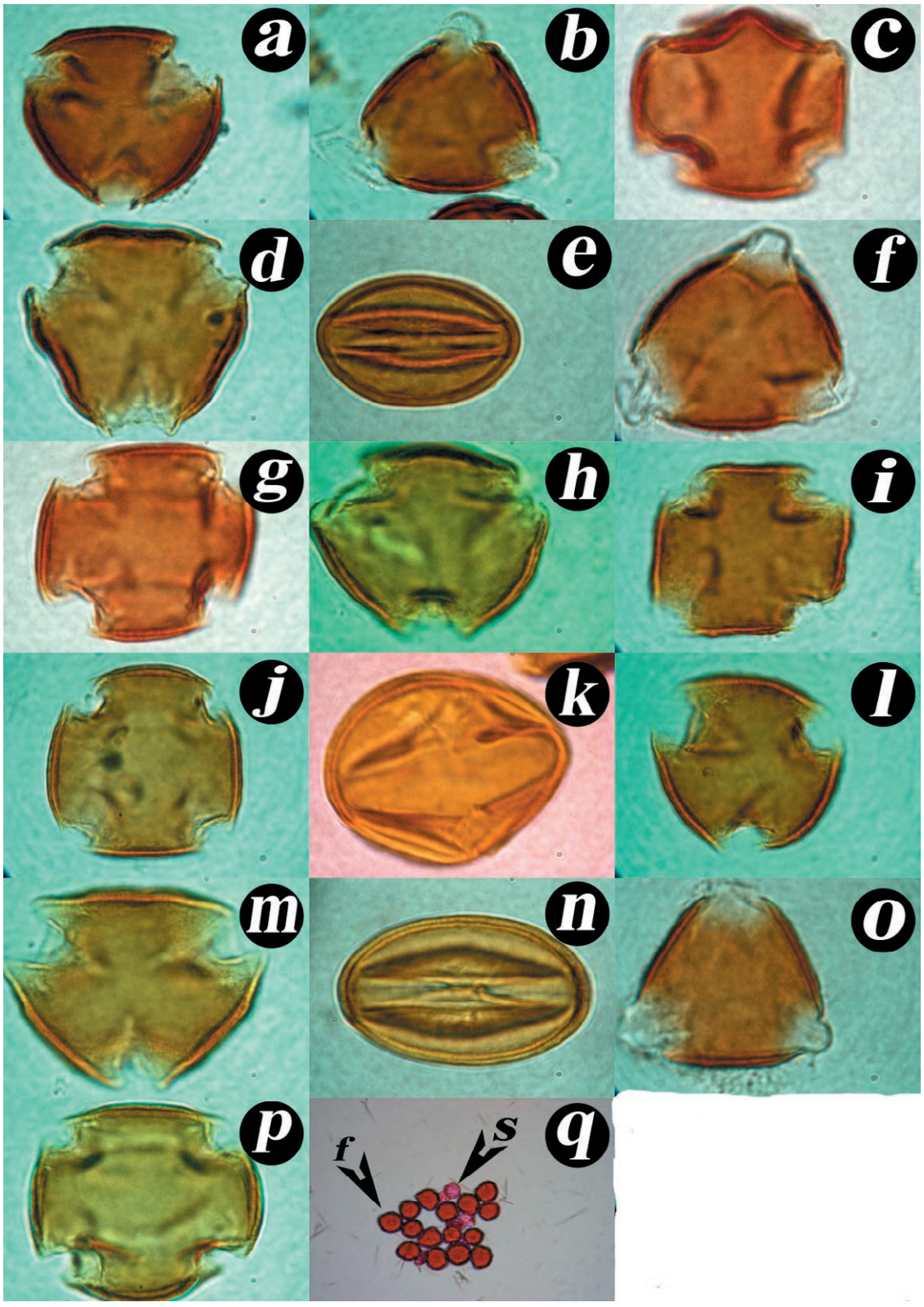

Figure 3. LM micrographs of pollen grains of *C. integerimus* (a), *C. melanocarpus* (b), *C. persicus* (c), *C. discolor* (d), *C. assadii* (e), *C. nummularius* (f), *C. esfandiarii* (g), *C. ovatus* (h), *C. hissaricus* (i), *C. turcomanicus* (j), *C. morulus* (k), *C. tytthocarpus* (l), *C. luristanicus* (m), *C. kotschyi* (n),*C. nummularioides* (o), *C. multiflorus* (p), fertil and steril pollen grain (q).

lel all over the surface except at the tubercles which are intermingled with together in these pearts (Figure 4. d).

C. morulus: The epidermal cells of the petal surface were loosely packed. Central part of each cell is raised into a fold surrounded by thick flat boundaries with distinct outline and rugose-tuberculate surface pattern which is condensed in the central fold or tubercle giving ruminate appearance. Cell margin is flat with smooth patterns (Figure 4. e).

C. tytthocarpus: The petal surface of this species exhibits closely packed epidermal cells. The cell surface is raised into broad finger-like projections and more or less thick folds, forming tubercle in the middle of the cell. The surface as a whole is rugose but at the tubercle becomes ruminate (Figure 4. f).

C. luristanicus: The epidermal cells of the petal surface are closely packed without distinct outline, showing rugose tuberculate pattern. The cell surface is raised into irregular projection giving appearance of simple folds or V-shaped folds. Ruga are observed all over the surface running parallel to each other or intermingling at the tubercle (Figure 4. g).

C. turcomanicus: The epidermal cells of petal surface are closely packed with rugose-tuberculate surface pattern and distinct outline. The cell surfaces are raised into big regular projections giving appearance of folds (Figure 4. h).

C. nummularioides: The petal surface exhibits rugose-ruminate pattern. The epidermal cells are distinct and loosely packed with thin walls. The elevated radial walls also show smooth pattern. The central part of the cells is raised into small finger-like projection (Figure 4. i).

C. kotschyi: Petal surface of this species exhibits closely packed without distinct outline. The cell surface is raised into broad finger-like projections and more or less thick folds, forming tubercles in the mid of the cell. The surface as a whole shows rugose to striate pattern which is parallel all over the surface except at the tubercle where these are intermingled with together (Figure 4. j).

C. assadii: The epidermal cells of the petal surface are loosely packed with distinct outline, showing rugose tuberculate pattern. The surface between radial walls of each cell exhibit striate pattern, the central part of the cells is raised into small irregular projections giving appearance of folds. The striae are condensed and forming ruminate pattern on the folds (Figure 4. k).

C. nummularius: Petal surface is composed of closely packed cells. Surface of the cell is raised into finger-like to folded projections or tubercles. The surface as a whole is rugose but at the tubercle becomes dense ruminate and cell boundaries are not clear (Figure 4. l).

C. ovatus: Petal surface of this species shows the cell boundaries prominently, the surface exhibits finger-like projections with rugose pattern (Figure 4. m).

C. esfandiarii: The petal surface of this species exhibits closely packed epidermal cells. The cell surface is raised into broad finger-like projections or tubercles, sometimes flattened into folds. The surface as a whole shows striate pattern except the top of the projections or tubercles where the striate show parallel and ruminate pattern (Figure 4. n).

C. discolor: The petal surface of this species exhibit closely packed epidermal cells. The cell surface is raised into broad finger-like projections or tubercles, sometimes flattened into folds. The surface as a whole shows striate pattern except the top of the projections or tubercles where the striate are ruminate pattern (Figure 4.o).

C. persicus: Petal surface of this species is loosely packed with traceable cell boundaries. The epidermal cell appears to be polygonal with raised folds. The central part of the cells is raised into small semi-regular projection giving appearance of folds. Sometimes the ruga are condensed forming ruminate pattern on the folds (Figure 4. p).

Abaxial surface of petal:

On the abaxial surface of petal two basic types of ornamentation are seen:

1) The striate surface as a whole, parallel and does not show the cell boundaries prominently (This form is observed in: *C. melanocarpus, C. multiflorus*, *C. luristanicus, C. kotschyi, C. ovatus, C. discolor*) (Figures 5. A, b, g, j, m, i).

2) The hive-shape with four to seven-sided houses in this form the boundaries between cells are clear. This form can be seen in the rest of the species studied (Figures 5.c, d, e, f, h, k, l , n, p).

These decorations were probably immature decorations. Because in some species such as *C. esfandiarii*, there was an intermediate of these two forms, and in species such as *C. ovatus* and *C. discolor*, in different individuals, there was one of the two forms.

Infrageneric variation

Both clustering and PCA analyses of the *Cotoneaster* species studied produced similar groupings and therefore only PCA analyses tree characters are presented here (Figures. 6 and 7).

The result based on pollen morphological: In this plot (Figures, 6), it can be seen that the two *Hissarici*

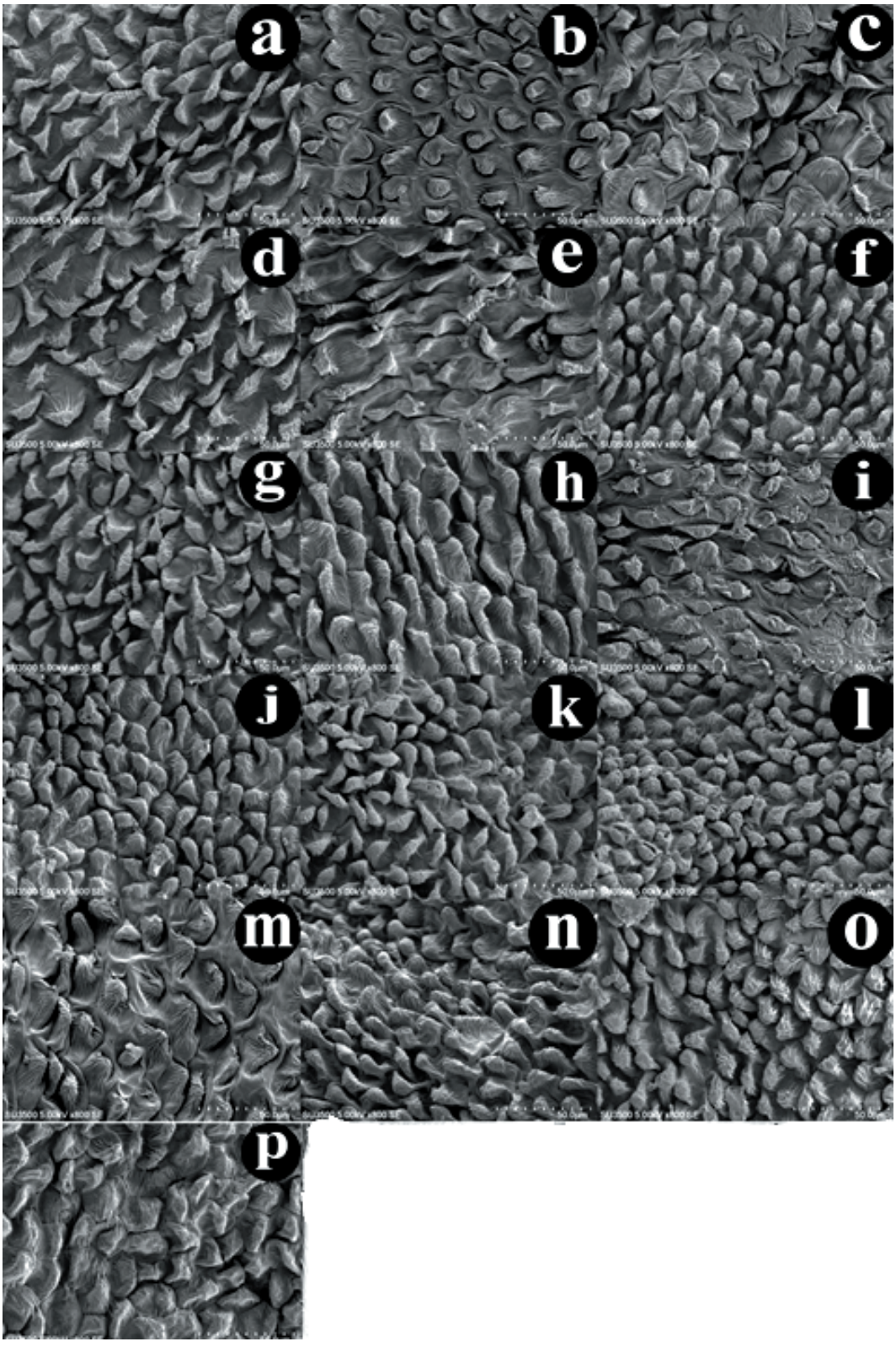

Figure 4. a-o. Micromorphological micrographs of ornamentation of adaxial surface in the studied species of *Cotoneaster*. *C. melanocarpus* (a), *C. multiflorus* (b), *C. suavis* (c), *C. hissaricus* (d), *C. morulus* (e), *C. tytthocarpus* (f), *C. luristanicus* (g), *C. turcomanicus* (h), *C. nummularioides* (i), *C. kotschyi* (j), *C. assadii* (k), *C. nummularius* (l), *C. ovatus* (m), *C. esfandiari* (n), *C. discolor* (o), *C. persicus* (p).

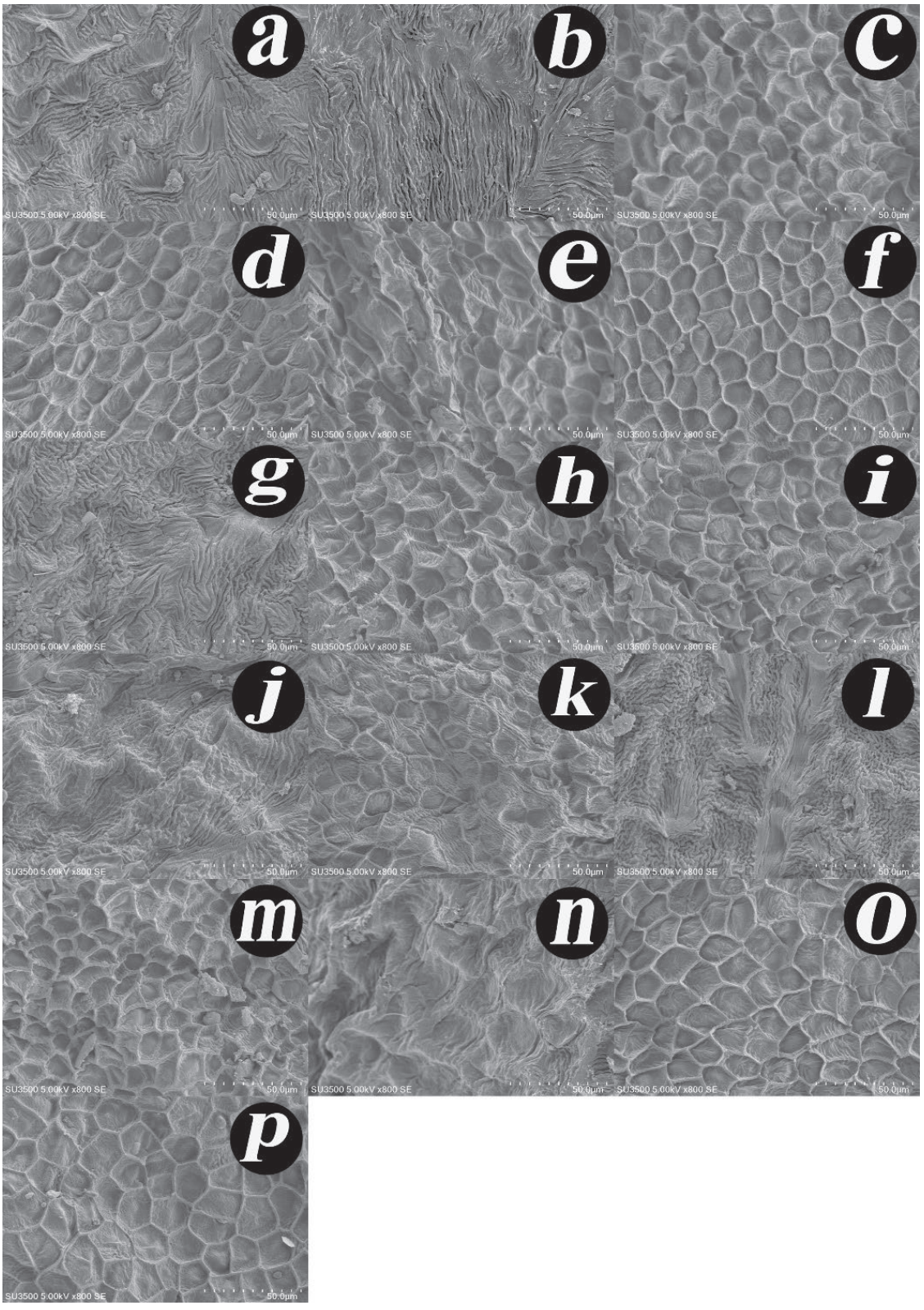

Figure 5. a-o. Micromorphological micrographs of ornamentation of abaxial surface in the studied species of *Cotoneaster*. *C. melanocarpus* (a), *C. multiflorus* (b), *C. suavis* (c), *C. hissaricus* (d), *C. morulus* (e), *C. tytthocarpus* (f), *C. luristanicus* (g), *C. turcomanicus* (h), *C. nummularioides* (i), *C. kotschyi* (j), *C. assadii* (k), *C. nummularius* (l), *C. ovatus* (m), *C. esfandiari* (n), *C. discolor* (i), *C. persicus* (p).

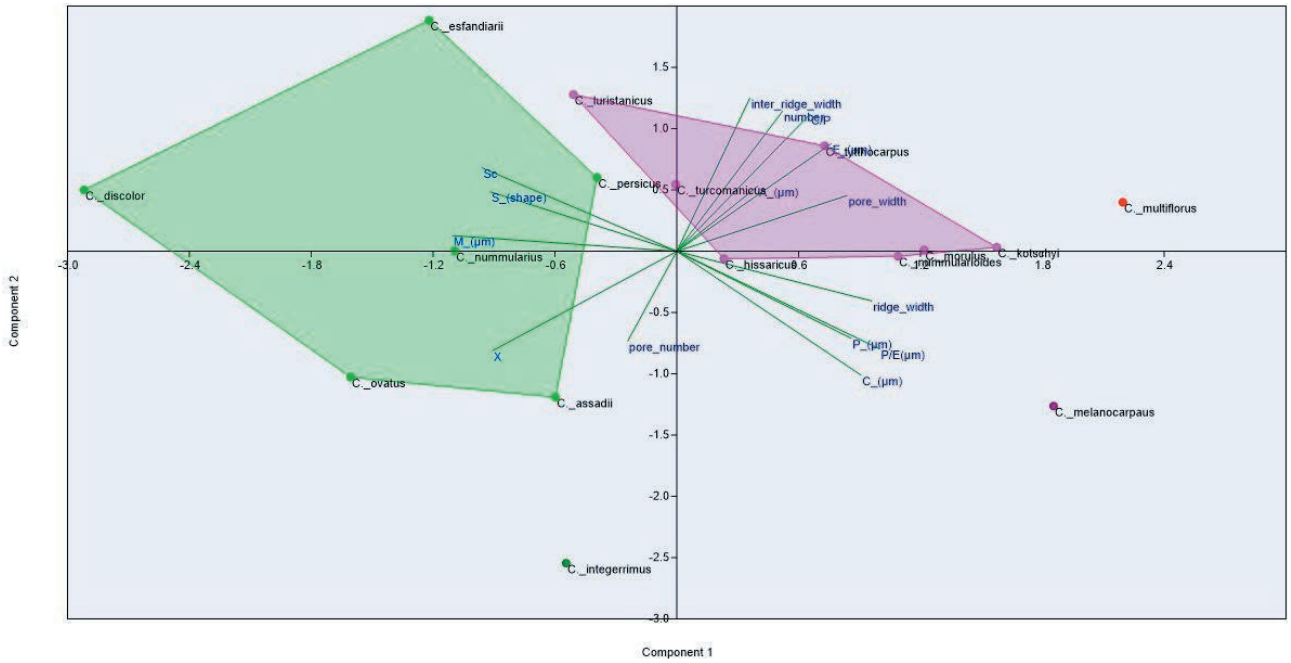

Figure 6. PCA plot of *Cotoneaster* species based on pollen morphological characters.

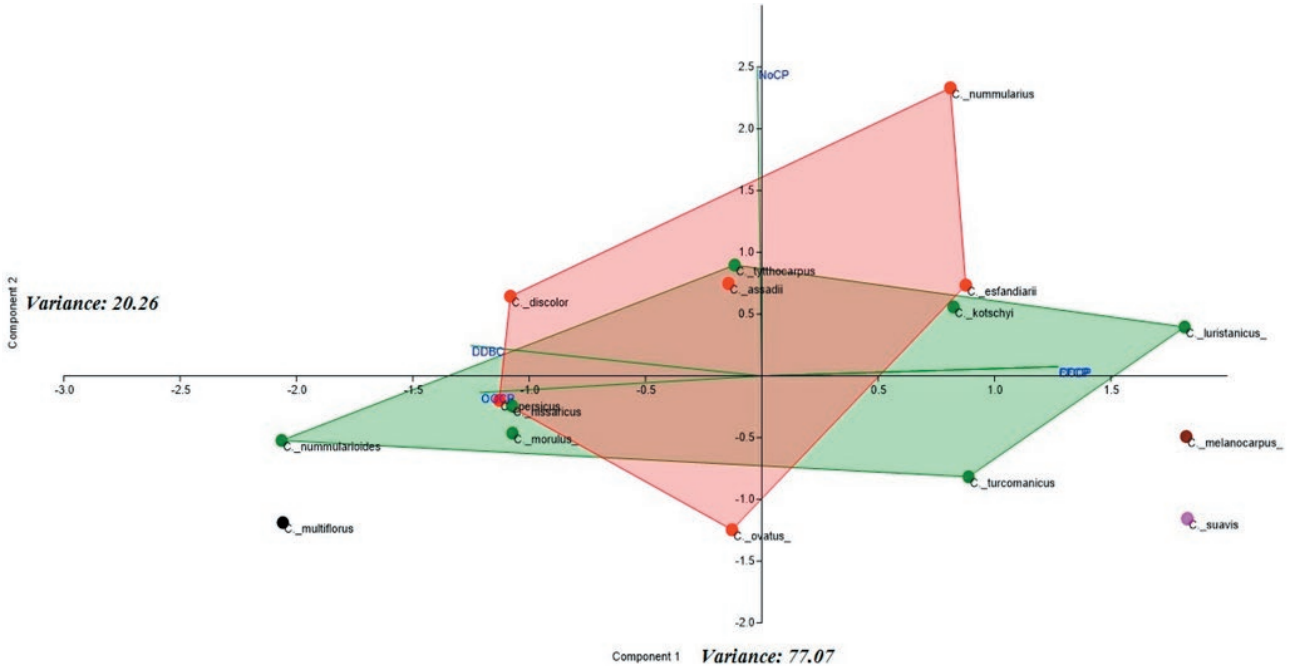

Figure 7. PCA plot of Cotoneaster species based on floral morphological characters.

and *Racemiflori* sections, which have the most species of this genus, are completely separated from each other and it can be said that using of pollen traits is probably effective in separating the sections and using these traits for placing a species in a particular section is probably helpful. The *Cotoneaster* subgenus members have considerable distance each other. *C. suavis* from the *Aitchisonioides* section and *C. multiflorus* from *Multiflori* sub-

section are also placed far from each other, which, due to the their few representatives in Iran, this separation cannot be interpreted as a meaningfull seperation. But, as we can see, the dispersal of the species in this chart indicates that pollen traits alone are not suitable for the separation, and that some species that are macromorphologically similar to each other, such as *C. discolor* and *C. persicus* are placed far from each other.

As shown in Figure 7, the first component variance is 77.07 and the second component variance is 20.26. The CCCP and FFCP traits have a significant positive correlation with the first component and the other three traits show a negative correlation with this component. Additionally, there is a significant positive correlation between the quantitative NoCP trait with the first component, and the remaining four qualitative traits show small positive and negative correlation with the second component.

Finally, the PCA analysis showed that petal traits in cotoneaster, as expected, are not separating traits, and the *Hissarici* and *Racemiflori* series species, which are the most common species in Iran, were overlapping in the terms of the separating petal traits. Subgenus *Cotoneaster* and some other series (*Multiflori, Aitchisonioides*), although they are separated, but because they have few representatives in Iran, it can be said that this separation is probably not meaningful and it can be relied only when more individuals of these subgenus, sections and series are studied.

DISCUSSION

Species delimitation and taxonomic consideration by pollen character

Many researchers have proven that taxonomic characters are of great interest for the correct identification of different plant groups (Ullah *et al*., 2018a; Ullah *et al*., 2018b).

The genus *Cotoneaster* like other tree and shrubby genera of *Rosaceae* such as *Amygdalus* L., *Pyrus* L., *Crataegus* L., *Rosa* L. is a morphologically difficult genus. Occurrence of hybridization which is a result of specific structure of flower, leads to appearance of individuals with intermediate characters. According to some studies on the family (Hebda and Chinnappa 1990) and also some genera such as *Amygdalus* (Vafadar *et al.* 2010), *Pyrus* (Xu and Yao 1990, Zamani *et al.* 2010), *Rubus* (Wronska- Pilarek *et al.* 2006), striate sculpturing is the predominant ornamentation in the family. An important feature in *Cotoneaster* different from other genera such as *Pyrus*, *Rubus*, *Amygdalus* and *Rosa* (Xu and Yao 1990; Wronska-Pilarek *et al.* 2006; Vafadar *et al.* 2010; Fatemi *et al.* 2012) is the presence of both tri- and tetracolporate pollen in the same specimen which is generally related to different levels of ploidy (Borsch and Wilde 2000).

On the basis of a comprehensive study on pollen morphology of the family *Rosaceae* in Canada (Hebda and Chinnappa 1990) it has been stated that variation in sculpturing is a diagnostic tool by which taxa can be identified, usually at the generic and often at the specific level. According to sculpturing, two main types striate (ridges and valleys) and non-striate (mainly psilate and verrucate) were recognized in the family (Hebda and Chinnappa, 1990). Moore (1991) has emphasized that pollen morphology in taxa of Rosaceae is very variable, even among the populations of the same species. Also, the grain size is the least reliable feature that is related to the comparatively frequent occurrence of hybridization in this family. This problem is remarkable in this study in the case of shape and sculpturing, even in different specimens of the same species. The importance of pollen morphological characters and their fitness for the most actual subgeneric taxonomic grouping are discussed in the following.

Subgen. *Cotoneaster.* ser. *Cotoneaster*

In this research, two species of subgenus *Cotoneaster* were studied. As shown in Table 2 and 3, the pollen characters in these two species are very similar to each other. This confirms the previous results that said that subgenus *Cotoneaster* is monophyletic (Li *et al.* 2014). In addition, because the pollination of this subgenus is highly dependent on a particular group of bees, the similarity of pollen grains in this subgenus can be evolutionary.

Subgen. *Chaenopetalum.* ser. *Hissarici*

Similar ornamentation pattern in *C. nummularioides* and *C. kotschyi* and dissimilarity from others are in line with other similarities between these species (including subcoriaceous, small (15 × 13 mm) and ovate or broadly elliptic leaves, compact inflorescence and number of flower (2 -5) per inflorescence, black and small fruits, navel open and also the same geographical distribution in Iran). *C. hissaricus* and *C. tytthocarpus* are very similar to each other in having similar morphological (size and shape of leaves, color and size of fruit, villose and depressed calyx, open navel) and pollen characters (P/E, type of sculpturing, shape of pollen) which distinguish these taxa from

other species of the series. *C. hissaricus* comes from Tajikistan and Afghanistan and *C. tytthocarpus* occurring in Tajikistan. Also, both of species are tetraploid (Fryer & Hylmo 2009). Morphological, micromorphological characters of pollen, origin center and chromosome number suggest they could be regarded as related species, although their area of distribution in Iran is not the same (first species distribution is in NW Iran and latter is in NE Iran). The pollen morphology in *C. morulus* is very heterogenus, the exine having variable sculpturing type, and does not provide much useful information for the interspecific delimitation within the series *Hissarici*.

Subgen. *Chaenopetalum*. ser. *Racemiflori*

Widely distributed *C. ovatus* (with ovate leaves and red fruit) and *C. assadii*. (with obovate leaves and red – orange fruit) are considered closely related by the similar ornamentation, polar axis length, pore number in area unit, inter ridge width and apocolpium length. With respect to their similar macromorphological characters (size of leaves and fruits, number of flowers in per infloresens, habit of plant) and distribution area, the overlapping pollen morphologies of *C. ovatus* and *C. assadii* provide support for the same origin of these species. According to Khatamsaz (1993), *C. esfandiarii* is placed in the *Cotoneaster* subgenus (by erect petals and 2-3 style) but Fryer and Hylmo placed it in the *Chaenopetalum* subgenus, *Racemiflori* section (by spread petals and 2- 3 style). In this survey, based on the exine sculpturing, *C. esfandiarii* resembles members of subgenus *Chaenopetalum* more to species of subgenus *Cotoneaster*, but the judgment in this case requires further studies. *C. persicus* and *C. discolor* are much alike in their pollen ornamentation. *C. discolor* pollen (with obscure ridge) differs from that of *C. persicus* (psilate sculpture) usually by having of a few number perforations in area unit.

Many of these species are relatively specific in their habitat requirements on the dry slopes (e.g. *C. persicus*, *C. prunoisus*, *C. kotschyi*) or wet regions (*C. assadii*) and may prove to be important habitat indicators. Also, the presence of pollen grains of these species in the depths of a region can be partly informed of the climate of that area in a particular geological period.

In conclusion, our findings revealed the palynological characteristics (e.g., perforation number, size and exine sculpturing) of the genus *Cotoneaster*. The similarity of exine structure and ornamentation, as well as the similarity of the various parameters analyzed at interspecific level makes it hard to establish taxonomical boundaries and clearly shows the affinity of species as far as morphological characteristics are concerned.

Species delimitation and taxonomic consideration by micromorphological petal

Taxonomic perspective

Shaheen *et al.* (2016) analyzed the shape of petal epidermal cells and their wall patterns within *Rosaceae* and concluded that family had a high degree of petal micromorphological variation, but we found only little differences among *Cotoneaster* species. Our result showed that there was not a significant variation at interspecific level in the 16 studied species. Unlike other genera of the Rosaceae, e.g. *Rubus*, *Crataegus* (Christensen 1992; Christensen and Hansen 1998; Sharifnia & Behzadi Shakib 2012; Hamzeh'ee *et al.* 2014), *Sibbaldia* (Tahir and Rajupt 2010) and *Rosa* (Sharma *et al.* 2005), *Cotoneaster* species petals decorating the microscopic level, did not show significant variation (exception number of conical projections). The petal epidermal features among species were fairly similar to each other. The shape of petal cells in the all of species was conical to finger-shape projection on the adaxial side. Conical cells may increase petal brightness and therefore increase pollinator visitation rates (Glover and Martin 1998; Comba *et al.* 2000; Dyer *et al.* 2006; Ojeda *et al.* 2009). The micromorphological properties of petal surfaces showed some variations. Number of conical projections is an important diagnostic character. The abaxial epidermis surface of these petals had a uniform pattern and cells with different sizes joined together in a fixed pattern.

Asexual seed production or apomixis, which is often associated with hybridization and polyploidy (Marshall & Brown 1981; Nogler 1984), has been reported in five Maloid genera e.g. *Amelanchier* Medik. (Campbell *et al.* 1985) and *Cotoneaster* (Hjelmqvist 1962). Such plants will therefore produce some completely maternal progeny through apomixes (Stebbins 1950). Consequently, apomixis genes can be much older than the clones they are currently contained in (Van Dijk 2003). Apomixis also has been reported frequently in *Cotoneaster* (Rothleutner *et al.* 2016). Since some of the maternal traits can be preserved for a long time through apomicies (Stebbins 1950), one of the reasons for not changing the ornamentation of the adaxial surface of the petals and their similarity to each other in different species is apomixis. The interesting thing is that micromorphological traits of petal in two species *C. hissaricus* and *C. morulus*, very similar to each other. These two species have similar macromorphological characters (shape and size of leaf, size and color of flower, shape and color of fruit) and regional distribution in Iran (Azerbaijan province).

Evolutionary perspective

Pollination is done by bees in *Cotoneaster* mainly by the short-tongued bumble bees (*Bombus terrestris* and *bombus lucorum*) and honey bees (*Apis mellifera*) which visited species in both subgenera of the genus, concentrating on the subgenus *Cotoneaster* during early summer and on *Chaenopetalum* after mid-June. The section *Cotoneaster* is recommended as particularly valuable for bee forage. Plants of the subgenus *Cotoneaster* were visited more by these bees in May and early June, a critical period when other forage may be scarce. The common carder bee *(Bombus pascuorum)* and the early bumble bee (*Bombus pratorum*) almost exclusively visited plants in the subgenus *Cotoneaster* throughout the season (Corbet *et al.* 1992). These findings and similar studies suggest that pollination of *Cotoneaster* and bee nutrition strongly linked together (Toth *et al.* 2011). Also, the *Cotoneaster* petals are white (especially in the section *Chaenopetalum*), for this reason the petal cell ornamentation on the adaxial surface is very important in attracting bees. The periclinal wall pattern of petal cells in all species studied is conical. Different species of *Cotoneaster* have the same pollinators and therefore there is not much difference between the adaxial surface ornamentation of the petals. Previous research has shown that flowers and their pollinators in many plants evolve together and has suggested that the rise of bees coincided with the largest flowering plant clade, the eudicots (Cappellari *et al.* 2013). Probably, pollination by certain species of bees is the only way to reproduce sexually in *Cotoneaster* and for this reason, the various species of this genus have evolved with each other in terms of petals, along with the particular species of this bee.

Ecological perspective

It seems that petal traits are stable in different species of this genus and do not change under the influence of the ecological conditions. Because all species that have been collected from different climates of Iran have almost the same ornamentation in their petals. As can be seen, in species with long and open inflorescences that have large flowers, the number of conical projections per unit area is lower and the boundary between the cells is quite distinct (*C. melanocarpus, C. multiflorus, C. suavis, C. ovatus*). This form of inflorescence and flower is found in species that have large, thin, and crusty leaves. On the other hand, these leaf traits are seen in mesophytic species. Thus, the high density of papillae on the adaxial surface of the petals can be a reason to deal with the dryness of the air. So mesophytic species do not have a high density of papillae. It is important to note that species with same traits, may also be found in semi-arid regions (*C. suavis, C. ovatus*). It can be concluded that petal traits maybe have been evolved once at the time of splitting species of this genus and in subsequent periods they have not been changed under the climate differences (similar results are obtained for leaves in the species of this genus).

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