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Characterization of nuclear DNA content and chromosome numbers of *Tulipa luanica* Millaku, *T. kosovarica* Kit Tan, Shuka & Krasniqi and *T. albanica* Kit Tan & Shuka

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Abstract: The Balkan Peninsula is considered an important centre of native tulip species. *Tulipa kosovarica* and *Tulipa luanica* are new species recently discovered in Kosovo, and *Tulipa albanica* in Albania. The current study aims at the investigating the nuclear DNA content and chromosome number of these three tulipa species in order to provide for the first time data on their genome size and differences among these three *Tulipa* species. Analysis of nuclear DNA content was performed by flow cytometer (Partec CyFlow Space) in mature fresh leaves for each *Tulipa* species. Samples for chromosome analysis were taken from the root tip meristem of the bulbs. Results showed significantly higher amounts of nuclear DNA (2C) in *T. luanica* compared to *T. kosovarica* and *T. albanica*. The chromosome number for these three species was 2n = 2x = 24, while the chromosome sizes of *T. luanica* resulted larger, compared to that of *T*. *kosovarica* and *T. albanica*. A correlation between the nuclear DNA content and chromosome size was found among these tulipa species. Moreover, nuclear DNA content and chromosome sizes of *T. luanica, T. kosovarica* and *T. albanica* showed clear differences among these species.

Key words: tulip; DNA content; chromosome number; endemica

Določitev vsebnosti jedrne DNK in kromosomskega števila treh vrst tulipanov, *Tulipa luanica* Millaku, *T. kosovarica* Kit Tan, Shuka & Krasniqi in *T. albanica* Kit Tan & Shuka

Izvleček: Balkanski polotok je pomemben center samoniklih vrst tulipanov.Vrsti *Tulipa kosovarica* in *Tulipa luanica* sta novi vrsti nedavno odkriti na Kosovu, in vrsta *Tulipa albanica* v Albaniji. Namen raziskave je bil preučiti vsebnost jedrne DNA in kromosomskega števila teh treh vrsta tulipanov in tako prvič določiti velikost njihovega genoma in razlike med temi tremi vrstami. Analiza jedrne DNA je bila narejena s pretočnim citometrom (Partec CyFlow Space) v odraslih svežih listih vseh treh vrst. Vzorci za analizo kromosomov so bili vzeti z rastnih vršičkov korenin čebulic. Rezulatati so pokazali značilno večjo vsebnost jedrne DNA (2C) pri vrsti *T. luanica* v primerjavi z vrstama *T. kosovarica* in *T. albanica*. Kromosomsko število vseh treh vrst je bilo 2n = 2x = 24, med tem, ko je bila velikost kromosov vrste *T. luanica* večja v primerjavi z velikostjo pri vrstah *T. kosovarica* in *T. albanica*. Pri vseh treh vrstah je bila ugotovljena korelacija med vsebnostjo jedrne DNK in velikostjo kromosov. Vse tri vrste so se po vsebnosti jedrne DNK in velikosti kromosov jasno razločevale.

Ključne besede: tulipan; vsebnost DNK; kromosomsko število; endemit

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1 INTRODUCTION

The genus *Tulipa* includes the most well-known, adored, and economically significant flowering plants in the world. In the World Checklist for *Tulipa*, 418 are named and 112 were accepted there (Govaerts 2008). Tulips species are grown in wild in North Africa, Southern Europe, the Middle East, and Central Asia, including China. According to Botschantzeva (1962), the Tien Shan and Pamir-Alay Mountain ranges in Central Asia are considered as primary centers while the Caucasus as a secondary gene center for *Tulipa* species. In the Balkan peninsula Greece, Kosovo, Bulgaria, Albania, Macedonia and Serbia have the highest number of tulip species (Govaerts, 2010; Millaku et al., 2018). Up to date in Kosovo there are reported five native tulipa species, *T. serbica* Tatić & Krivošej*, T. australis* L.*, T. gesneriana subsp. T. scardica* L.*, T. luanica* Millaku and *T. kosovarica* Kit Tan, Shuka & Krasniqi (Millaku & Elezaj, 2015; Shuka et al., 2010, 2012). While the *Tulipa* genus is represented by two stenoendemic species, *T. kosovarica* and *T. serbica*, which are found only in the serpentines of Kosovo. *T. scardica* and *T. kosovarica* grow in serpentine soil, well-drained and exposed to full sun but protected from high winds, while *T. luanica* grows in limestone soil (Osmani et al., 2018). These serpentine soils were characterized by high concentrations of metals, and the levels of metals such as Ni, Co and Cr ranged from 1500 to 1600 mg kg^{-1} for Ni, 130 to 140 mg kg^{-1} for Co, and 380 to 450 mg kg^{-1} for Cr (Osmani et al., 2018). This previous study reported that the enzyme δ-aminolevulinic acid dehydratase activity and concentrations of δ-aminolevulinic acid, malondialdehyde and glutathione showed differences among these three tulipa species, especially *T. kosovarica* and *T. albanica* (serpentine sites) in comparison with *T. luanica* (limestone site).

Despite the existence of a large body of literature on *Tulipa*, taxonomy is generally considered to be difficult. Moreover, molecular analysis is a good attempt to understand the relationships within *Tulipa* species better. Nuclear DNA content can conveniently be measured by flow cytometry using propidium iodide, a stoichiometric DNA stain that intercalates in the double helix (Zonneveld, 2009). It is well known that morphological characteristics of plants are influenced by the vegetative stage of the plant as well as by a variety of environmental conditions. Therefore, the identification of the genetic diversity within and among plants species based on the morphological plant characterization is considered insufficient (Hunter, 2018). DNA-based markers are an advanced tool widely used to assess genetic relationships and diversity in plant species (Kumar, 1999; Hunter, 2018), novel molecular marker techniques have been developed in diverse plant species (Wang et al., 2015) and the transferability of molecular markers between species has been investigated for many species so far (Raveendar et al. 2015; Berisha et al. 2015). The recent advances in sequencing technologies enabled the discovery of functional genes in many plant species (Abbasi et al., 2015; Chai et al., 2017). Furthermore, these initiatives have enabled the development of novel and alternative molecular markers known as gene-targeted markers (GTMs), which are based on the untranslated sections of expressed sequence tags (ESTs) (Poczai et al., 2013) or gene-targeted functional markers (GTFMs), which are gene markers implicated in phenotypic trait variation as a result of their functional gene sequences (Arnholdt-Schmitt, 2005). In addition of above mentioned techniques, flow cytometry helps in the estimation of nuclear DNA content and the ploidy level (Dolezel et al., 2021; Dolezel et al., 2004; Vlacilova et al., 2002). Flow cytometry is a method that can conveniently measure the nuclear DNA content by using propidium iodide, a stoichiometric DNA stain. A genus may contain numerous species with similar chromosomal numbers but different DNA 2C-values (Ohri, 1998). Flow cytometry is a fast and practical method for elucidating systematic relationships among species within the genus. This technique was efficiently employed in ecological, physiological, molecular biology and genome evolution studies, as well as in plant breeding (Dolezel et al., 2021).

The chromosomal karyotype parameters of Iranian *Tulipa* species were studied by Abedi et al. (2015) and Masoud et al. (2002). Their findings demonstrated that while the majority of Iranian species had three distinct chromosome types—m, sm, and st—and were diploid (2n = 2x = 24), their karyotype parameters varied**.** In the Netherlands, the genome size of a variety of *Tulipa* species was studied with results ranging from diploid (30 pg) to tetraploid (123 pg) (Zonneveld, 2009).

Based on our previous investigation, *T. kosovarica* and *T. albanica* grow on serpentine soils while *T. luanica* grows in limestone soil (Osmani et al., 2018). The serpentine soils were characterized by higher concentrations of metals compared with limestone soils. Since these three endemic species are relatively new species discovered in recent years and they grow in different habitats, traditional taxonomy based on geographic distribution and morphological characteristics will be augmented with more information on DNA content and chromosome number to clarify the relationships among these *Tulipa* species. The main objective of this study was to determine nuclear DNA content and chromosomes number of *T. kosovarica*, *T. luanica* and *T. albanica,* and in line with this to have more information about differences between these species.

2 MATERIAL AND METHODS

The plant samples were collected during flowering time in their natural habitats (Figure 1): *Tulipa albanica* at Surroi locality in Albania (altitude 625 m a.s.l., geographical coordinates: 42°02'30" N and 20°20'15" E), *Tulipa kosovarica* at Mrasor locality in Kosovo (altitude 450 m a.s.l, geographical coordinates: 42°30'59" N and 20°34'08" E) and *Tulipa luanica* at Pashtrik locality in Kosovo (altitude 1100 m a.s.l, geographical coordinates: 42°16'17" N and 20°28'24" E). More than 30 plant samples (leaves and bulbs) from each plant species were collected. The sample collections and preparation procedures were carried out at a regulated temperature of 4 °C.

2.1 FLOW CYTOMETRY ANALYSIS

FCM (flow cytometry) is a great method for examining the optical characteristics of small particles suspended in liquid, such as fluorescence and light scatter. The nuclear DNA content and polyploidy analysis of our sample set was carried out using Partec CyFlow Space flow cytometer (Munster, Germany) at analyzes the laboratory of genetics and plant cytogenetics at Namik Kemal University in Tekirdag, Turkey. Fresh mature leaves of three tulip species were collected during two seasons (2016 and 2017) during their flowering time in their natural habitats and were analyzed within the optimal time frame after their collection. The collection, storage and transport of leaves was carried out based on standard procedure and under temperature control (4 °C) with preservation of humidity. The analysis of nuclear DNA content was done with three replicates within the sample (from the same leaf) and from 30 plant individuals (leaves), at least 5000 nuclei were analyzed for each type and sample. Suspension of intact nuclei was prepared using commercial kits manufactured by Partec (Munster, Germany). Homogenization of the leaves (50 mg) was done together with the leaves of the standard plant in a petri dish, where 0.5 ml of extraction buffer was also added. This homogenate was filtered using 50 µm nylon filters and then transferred to standard test tubes of the apparatus where 1.5 ml of DAPI (4',6–diamidin–2-phenylindole) was added; samples were left in the dark for 60 minutes at 4 °C. (Tuna et al., 2001). DAPI was used as a fluorochrome for DNA labeling because it has more affinity for binding to the nitrogenous bases A and T. *Secale cereale* L., which has 16.55 pg / 2C DNA, was used as a standard. The results were processed with the FloMax analysis software program and expressed in pg 2C DNA (picograms of diploid DNA) (Figure 2). The amount of nuclear DNA was calculated based on the following formula:

2C DNA = [(average G1 peak of sample / average G1 peak of standard)] × amount of 2C DNA standard (pg DNA)

2.2 KARYOTYPE ANALYSIS

Samples for chromosome (karyotype) analysis were taken from the root tip meristem of the bulbs. First, the bulbs were collected from their natural habitats, and then they were grown in a vegetative room. After germination, approximately 2 mm root tips were cut and treated with 0.05 % colchicine for 5 hours, then fixed with acetic acid/ethanol in a ratio of 1:3 for 24-48 hours. Hydrolysis of

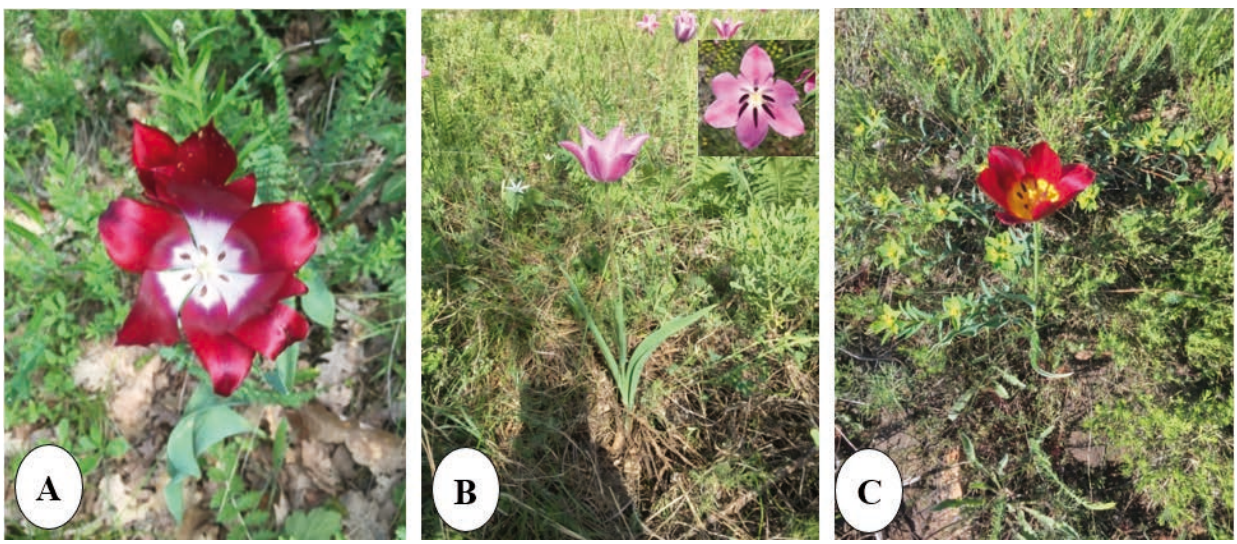

Figure 1: Tulipa species during flowering time in their natural habibitats: A) *T. kosovarica*, B) *T. luanica* and C) *T. albanica*

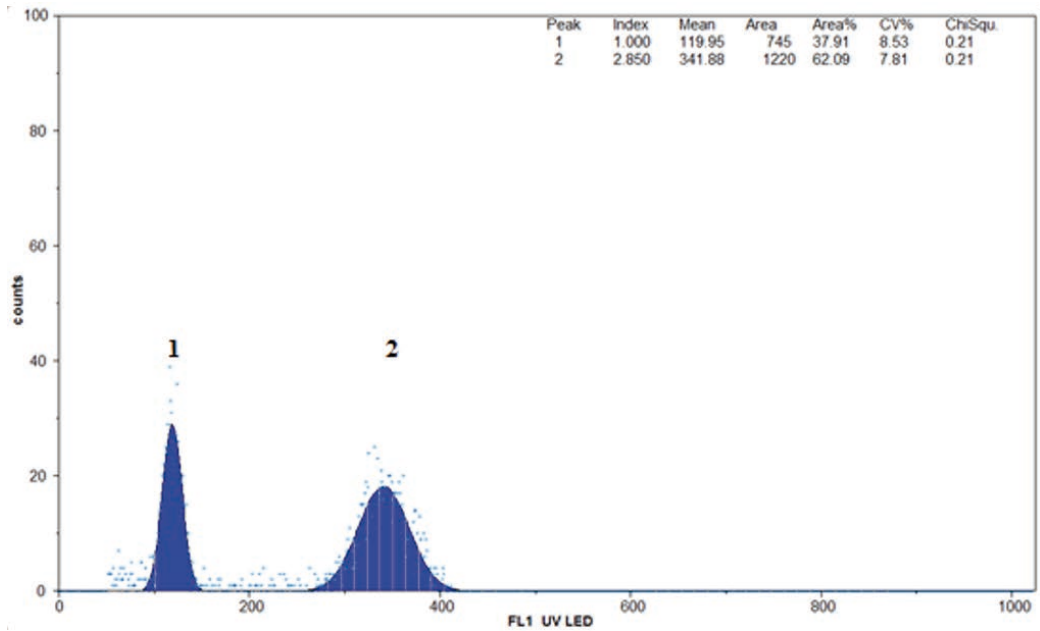

Figure 2: Flow Cytometer histogram for the amount of nuclear DNA (2C) in: 1) standard plant and 2) *Tulipa luanica*

the tip of the roots was done using 1N HCl for 12 minutes at a temperature of 60 °C, while staining was done by using acetocarmine. Karyotyping, determination of centromere position and chromosome type was performed in the metaphase according to the nomenclature of Levan et al. (1964).

2.3 DATA ANALYSES

Statistical analysis of the results was carried out with Sigma stat 32 programs 2004 STAT Software. The data presented in the paper represent the average of at least four independent experiments with ± S.E. Each continuous variable, a distribution form was determined, and the significant differences between means were checked by Student's *t* test.

3 RESULTS AND DISCUSSION

Seven different taxa of *Tulipa* are found in Kosovo, three of which are stenoendemic (*T. luanica, T. kosovarica,* and *T. serbica*), one of which is a local endemic (*T. scardica*), while the other three taxa, *T. gesneriana* and *T. sylvestris* which is represented by two subspecies (*T. sylvestris* subsp. *australis* and *T. sylvestris* subsp. *sylvestris*), have a wider distribution (Millaku et al., 2018; Millaku & Elezaj, 2015; Shuka et al., 2010, 2012). The presence of a high number of *Tulipa* species/taxa in Kosovo and their sympatric area in serpentine substrate in the Deva locality, in the south of Kosovo, close to the border with Albania, makes it an important regional and global habitat of native species of the genus *Tulipa*. According to Millaku et al. (2018), considering tulip species high variability, the application of molecular analyses is of crucial importance to accurately classify *Tulipa* species of Kosovo and of the Balkans and in their taxonomic differentiation.

3.1 NUCLEAR DNA CONTENT

The results indicate a significantly higher amount ($p < 0.001$) of nuclear DNA (2C) in *T. luanica* (47.49 pg) compared to *T. kosovarica* (45.71 pg) and *T. albanica* (43.86 pg) (Table 1). Based on differences in the amount of nuclear DNA expressed as a percentage, we can presume that *T. luanica* and *T. kosovarica* differ by 3.75 %, *T. luanica* and *T. albanica* differ by 7.64 %, while *T. kosovarica* and *T. albanica* differ by 4.05 %. Our results regarding the genome size (2C DNA) of *T. luanica*, *T. kosovarica* and *T. albanica* are in accordance with those reported by other authors; Zonneveld (2009) reported that the genome size of plants of the genus *Tulipa* sp. with diploid number (2n) of chromosomes varied from 32-69 pg 2C DNA. Our genome size results (2C DNA) showed a range of 43.86 – 47.49 pg in the three tulip species, which are within the values reported for tulips with diploid chromosome number by Zonneveld (2009). In our study the 2C DNA content of the *T. albanica* resulted 43.86 pg, while in the same speciesthe 2C DNA content was previously reported to be 54.15 pg (Shuka et al. 2010). The

observed differences in the genome size in the same tulipa species can be due to the fact that, in our analysis, we used adult leaves collected at the time of blooming from naturally grown plants, while according to Shuka et al. (2010), the plant material used for the amount of DNA estimation was taken both by the germinated seeds and the adult leaves. Fresh leaves that have nearly completed growth are often preferred; very young leaves might not be as suited because they contain more inhibitors than older leaves (Dolezel et al., 2007). The genome size (2C DNA) reported for other tulips that grow in the Balkans (Albania and Kosovo) was 61.5 pg in *T. schrenkii* Regel and 69 pg in *T. scardica* Bornm. (Zonneveld, 2009). If we compare species of the genus *Tulipa* that extend from east to west, from North Pakistan to the Balkans, a gradual increase in the amount of nuclear DNA is observed, from 32 pg to 69 pg (Zonneveld, 2009). The transfer of DNA sequences from the nucleus into mitochondria and chloroplasts may have been one of the causes for smaller genome sizes (Karimzadeh et al., 2010).

The results of the number of base pairs calculated using a value of 978 mega base pairs (Mbp) for one picogram, showed that *T. luanica* had a greater number of mega base pairs compared to the *T. kosovarica* and *T. albanica* (Table 1). Based on these results, *T. luanica* has about 1740 Mbp more than *T. kosovarica* and about 3550 Mbp more than *T. albanica*, while *T. kosovarica* has about 1809 Mbp more than *T. albanica*. Genome size results were also given with flow cytometer histograms for each tulip species. According to previous studieson genome size, 1pg is equal to several thousand genes or about 978 Mbp (mega base pairs) (Zonneveld, 2009; Dolezel et al., 2003). According to our results, *T. luanica* has a larger genome, a larger number of genes and base pairs, compared to other tulip species under study, *T. kosovarica* and *T. albanica,* the latest are grown on serpentine soils. According to Knight et al. (2005) and Temsch et al. (2010), large genomes are a burden for plant organisms and limit their adaptation. On this regards, plant species that grow under stressful habitats face greater risk; therefore, we presume that the two plant species *T. kosovarica* and *T. albanica* have adapted in terms of genome size to live in serpentine environments, which are considered potentially more stressful, while *T. luanica* in limestone environments has a larger genome.

3.2 CHROMOSOME NUMBER AND KARYOTYPE CHARACTERISTICS

Analysis of the results for chromosome number and karyotype characteristics for *T. luanica, T. kosovarica and T. albanica* are presented in table 2 and figure 4. Based on these results we can conclude that the number of chromosomes in the three species under study, *T. luanica*, *T. kosovarica* and *T. albanica,* is 2n = 2x = 24 (Figure 3). The size of the chromosomes in *T. luanica* ranged from 5 - 12 µm, where two pairs of chromosomes are metacentric (I and VIII), two pairs are submetacentric (X and XII) and the other eight are subtelocentric (II, III, IV, V, VI, VII, IX and XI). In *T. kosovarica* the size of the chromosomes ranged from 5 - 10 µm, where two pairs of chromosomes are metacentric (X and XII), five are submetacentric (II, IV, V, VI and XI) and the other five subtelocentric (I, III, VII, VIII and IX). In *T. albanica* the size of the chromosomes ranged from 5 - 8 µm, where two pairs are metacentric (V and VIII), three pairs submetacentric (VI, X and XII) and the other seven chromosomes are subtelocentric (I, II, III, VI, VII, IX and XI).

Our results for chromosome number and karyotype characteristics of *T. albanica* and *T. luanica* were in accordance with those reported by other authors (Shuka et al., 2010; Millaku & Elezaj, 2015). According to Zonneveld (2009), most of the species of *Tulipa* have the same basic chromosome number, 2n = 2x = 24. In addition, many species in this genus have the same chromosome number, differences in DNA 2C value, when present, have proven to be very effective in delimiting infrageneric

Table 1: The amount of nuclear DNA in picograms (pg) in the leaves of *T. luanica, T. kosovarica and T. albanica*, the difference being expressed as a percentage (%) between these species and the approximate number of mega base pairs (Mbp)

		2C ADN pg	Difference in %	Equivalent in Mbp
T. luanica		47.49 ± 0.53	TL : TK = 3.75 %	46445.22
T. kosovarica		45.71 ± 0.29	TL : TA = 7.64 %	44704.38
T. albanica		43.86 ± 0.59	TK : TA = 4.05 %	42895.08
Significance	TL : TK	< 0.001		
	TL : TA	< 0.001		
	TK : TA	< 0.001		

TL – T. luanica; TK – T. kosovarica; TA – T. albanica. The results are expressed as mean and standard error (±). Significant differences were found between different species for $p < 0.001$

Table 2: Number of chromosomes and karyotypes of *T. luanica*, *T. kosovarica* and *T. albanica*

Species	Chromosome number	Chromosome size	Karyotype characteristics		
			Metacentric	Submetacentric	Subtelocentric
T. luanica	24	5-12 µm	I, VIII	X, XII	II, III, IV, V, VI, VII, IX, XI
T. kosovarica	24	5-10 µm	X, XII	II, IV, V, VI, XI	I, III, VII, VIII, IX
T. albanica	24	5-8 µm	V, VIII	VI, X, XII	I, II, III, VI, VII, IX, XI

divisions in a number of taxa (Ohri, 1998). Genome size has been demonstrated to differ between taxa that share identical chromosome numbers. Moreover, Greilhuber (1998, 2005) has clearly shown that intraspecific variation of genome size is much less than assumed. In this case, results show that the size of the genome (2C DNA) and chromosomes of *T. luanica* are larger compared to *T. kosovarica* and *T. albanica*. A correlation was also found between the 2C DNA content and chromosome size; the larger the genome, the larger are the chromosomes in these three types of tulips. In addition, morphological differences between these species reported from Millaku and Elezaj (2015) show that capsule and seed size of *T. luanica* are bigger in comparison with *T. kosovarica* and *T. albanica*. These findings are consistent with a considerable amount of evidences that suggest that the size of reproductive organs might be related to genome size and that the variations in genome size, both increases and

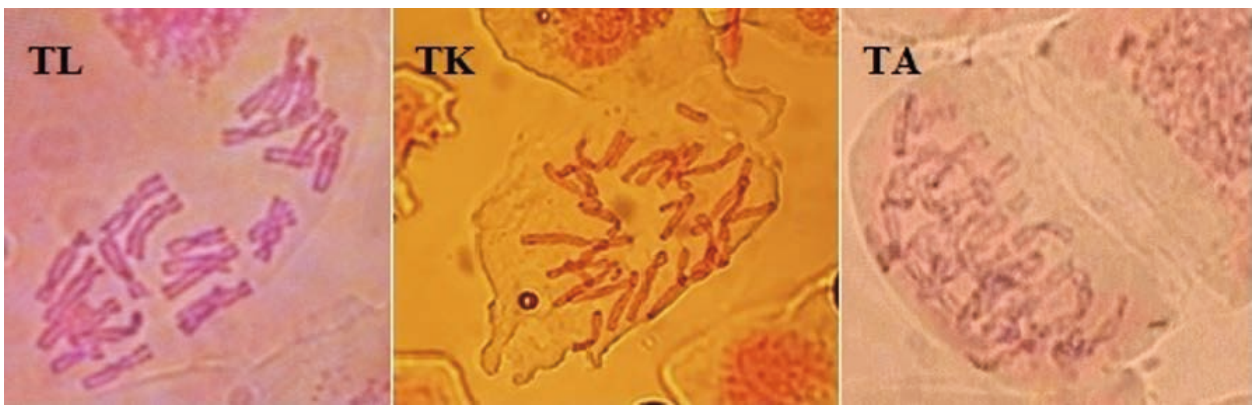

Figure 3: Karyotyping of chromosomes in metaphase for *T. luanica* (TL), *T. kosovarica* (TK) and *T. albanica* (TA)

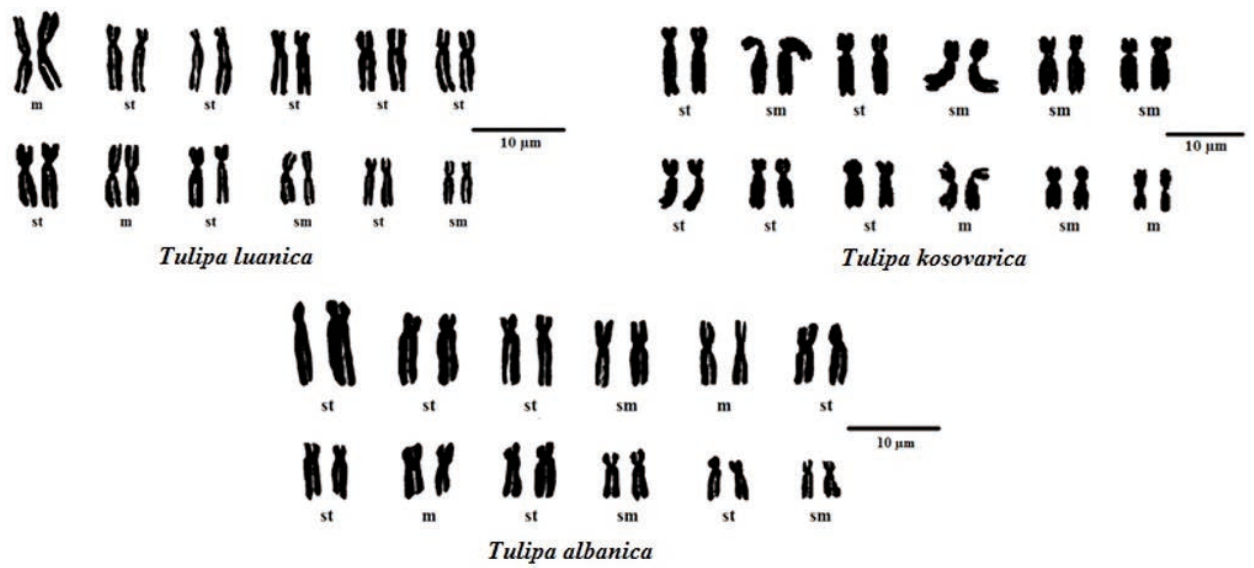

Figure 4: Karyogram and presentation of chromosomes in metaphase

decreases, might have contributed to the evolution and diversification of the genus, even within closely related species (Seijo & Fernandez, 2003).

4 CONCLUSIONS

The combination of analysis of nuclear DNA content carried out in flow cytometry and the number of chromosomes resulted very useful to determine the relationship status among three *Tulipa* species. Moreover, nuclear DNA content and chromosome sizes of *T. luanica, T. kosovarica* and *T. albanica* showed clear differences among these species. The nuclear DNA content and chromosome size of *T. luanica* resulted larger, compared to that of *T. kosovarica* and *T. albanica*. Furthermore, we presume that the two plant species *T. kosovarica* and *T. albanica* have adapted in terms of genome size to live in serpentine environments, which are considered potentially more stressful, while *T. luanica* which is grown in a less stressful limestone environment resulted in a larger genome size.

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