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Genetic diversity, population structure and chromosome numbers in medicinal plant species *Stellaria media* L. VILL.

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Abstract. *Stellaria media* L. VILL., is known under the name of chickweed, it is an annual plant in the family Caryophyllaceae. *Stellaria media* is distributed in the all regions of Iran and has been introduced to many habitats of the world. *S. pallida* is very similar to *S. media*. This plant is considered to be as a herbal remedy and is used in folk medicine. *Stellaria media* is edible and nutritious. In the present study, we used morphological and ISSR data for this species. For this, 43 morphological characteristics, including 16 qualitative and 26 quantitative. AMOVA and Gst analyses showed that the populations of this species are genetically differentiated. Nm analysis revealed very low value of genetic diversity among the studied population and mantel test indicated isolation by distance occurred among them. The present study showed that the studied populations of *S. media* are differentiated in morphological characteristics and genetic content. In general, species relationships obtained from morphological and molecular data were largely congruent.

Keywords. Genetic diversity, ISSR, Morphology, Species relationship, *Stellaria media*.

INTRODUCTION

The family Caryophyllaceae comprised about 81 genera and 2600 species (Bittrich 1993; Ullah *et al.* 2019a). *Stellaria* L. (Caryophyllaceae, Alsinoideae) includes both annual and perennial herbaceous plants that are widely distributed in the temperate zones of Europe and Asia (Lu and Rabeler 2001; Keshavarzi and Esfandani –Bozchaloyi, 2014a, 2014b; Ullah *et al.* 2019b, 2019c) and about 120 species with worldwide distribution, mainly in the north temperate zone (Morton 2005; Ullah *et al.*, 2018a, 2018b).

In Flora Iranica this genus has 9 species and divided into 2 sections: sect. *Pseudalsine* Boiss. consist of one species *S. alsinoides* Boiss. & Buhse and sect. *Stellaria* with six species: *S. holostea* L., *S. persica* Boiss., *S. graminea* L., *S. nemorum* L., *S. media* (L.) Vill., *S. pallida* (Dumort.) Pire (Rechinger 1988). Main center of diversification for *Stellaria* is Eurasia, with a center of distribution in the mountains of E. central Asia. Some species are also cosmopolitan (Bittrich 1993; Ullah *et al.* 2018c).

There are limited chromosome records for *Stellaria* in the world. Basic Chromosome numbers of $x=10, 11, 12$ and 13 have been reported for the genus (Federov 1969; Moore 1973; Goldblatt 1981). *Stellaria media*, chickweed, are annual and with slender stems, they have hairs on one side of the stem. The leaves are linear or oval, smooth or minutely, 13 to 17×1.5 to 7 mm. Flowers are hermaphrodite and petals are white with 5 deeply. Sepals prominently 4 to 6 -nerved, 4 to 7 mm. Stigmas are 3 and the stamens are 3 . *Stellaria media* common in waste places, open areas, lawns, meadows, and widely distributed to temperate regions of Europe, Asia and Northern America.

Stellaria media is edible and nutritious and has a history of herbal use and medicinal properties. This species has been used as to soothe severe itchiness even where all other remedies have failed (Slavokhotova *et al.* 2011). it is considered for rheumatic pains, skin diseases, and period pain as well as for bronchitis and arthritis (Slavokhotova *et al.* 2011). *Stellaria media* possess significant chemicals known as saponins, which can be cause saponin poisoning in cattle (Haragan 1991).

There are many studies which are on taxonomy, pollen morphology, phylogeny, seed micromorphology, anatomy, trichome and cytology of *stellaria* species (Esfandani-Bozchaloyi and Keshavarzi 2014; Keshavarzi and Esfandani-Bozchaloyi 2014 a, b; Ullah *et al.* 2018a, 2018b, 2018c). However, genetic diversity of *stellaria* species have been reported in a few studies (Verkleij *et al.* 1980; Chinnappa and Morton 1984), also outcrossing or inbreeding, genetic structure, genetic variability within/between populations and ecological adaptation on *Stellaria* of Iran have not been investigated yet.

According to Ellis and Burke (2007) genetic diversity are essential in the adaptability and survival of population, because it is as a way for adapt to changing environments in populations. The adapt of the population to

the changing environment will depend on the presence of the genetic diversity. Large populations have higher genetic diversity due to more to maintain genetic material and small populations have the loss of diversity which is called genetic drift. Mating or inbreeding between individuals with similar genetic occur in small population sizes, thus decreasing genetic diversity and finally we have more common alleles.

Hence, the used of markers will depend on the type of the species. We have been used DNA marker based techniques such as Inter-Simple Sequence Repeats (ISSRs), due to easy, highly reproducible, stable and useful in species delimitation, gene tagging, gene flow, breeding programs and evolutionary biology (Ellis and Burke 2007; Esfandani-Bozchaloyi *et al.* 2018a, 2018b, 2018c). Therefore, we studied morphological and molecular study of 11 geographical populations of *S. media* for the first time in Iran.

MATERIALS AND METHODS

Morphological studies

85 plant sample were selected from eleven populations located in three provinces of Iran. Identification of species *Stellaria media* were based on the descriptions provided by Flora Iranica (Rechinger 1988). The sampling sites and herbarium number are provided in Table 1, Figure 1. Vouchers were deposited at the herbarium of Islamic Azad University, Science and Research Branch, Tehran, Iran (IAUH).

DNA extraction

Fresh leaves of 85 individuals following a modified CTAB protocol. The quality was checked on a 1% agarose

Table 1. Location addresses and ecological characters of the *Stellaria media*

Population	Locality	Latitude	Longitude	Altitude (m)	Voucher no.
1	Guilan, Road to Sangar	37°06' 57"	49°11'06"	47	IAUH 201600
2	Guilan, Bandar Anzali, Pine artificial woodland	37°27'34"	49°42'40"	-25	IAUH 201701
3	Guilan, Loleman	37°28'59"	49°33'45"	-29	IAUH 201702
4	Guilan, Siahkal, Sangar	37°09'08"	49°55'02"	27	IAUH 201603
5	Guilan, Gole rodbar river	37°10'05"	49°56'38"	15	IAUH 201604
6	Guilan, Sheytan kouh hill side	37°12'04"	50°03'12"	9	IAUH 201605
7	Guilan, Lahijan, Highlands of Sheytan Kouh	37°11'52"	50°03'17"	159	IAUH 201606
8	Guilan, Bandar Anzali, Road side	37°27'48"	49°22'30"	-11	IAUH 201707
9	Mazandaran, Chalos Neamat abad	36°49'02"	50°52'20"	-16	IAUH 201608
10	Mazandaran, Shirodi Ring Road	36°51'10"	50°32'11"	-18	IAUH 201709
11	Mazandaran, Noshahr	36°35'04"	51°35'14"	-20	IAUH 201710

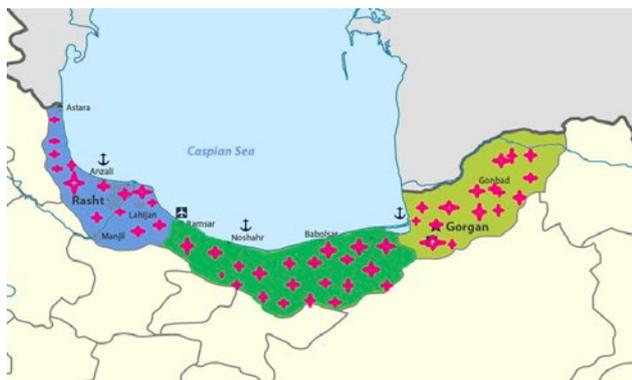


Figure 1. Distribution map of the studied populations.

gel and spectrophotometry. A set of ten primers; (GG) 5GT, (AA) 7GT, (AAA) 5Gt, UBC 234, (GG) 7AT, (AG) 7G, UBC 825, UBC 823, (GG) 5T and (GC) 8GG were used for ISSR analysis. PCR were carried out in 25 μ l reactions containing 20 ng of template DNA, 0.3 mM dNTPs, 1 μ M primers, 1.0 μ l of 20 \times PCR buffer (Cinnagen, Iran), 1.8 mM of MgCl₂, and 5 units of Taq polymerase (Cinnagen, Iran).

The amplification was carried out, with programmed as initial pre-denaturation at 95°C for 5 min followed by 36 cycles of denaturation at 94°C for 45 s, annealing at temperature (52-55°C) for 40 s, and extension at 72°C for 1min. A final 5 min extension at 72°C followed the completion of 38 cycles.

Karyological study

For somatic chromosome study, the seeds were soaked for 24 hours in running water and germinated in the laboratory (ca. 21°-24°). The root tips were cut between 9-11 AM and pretreated in 0.002M 8- hydroxyquinoline (4hours) and fixed in a cold mixture of ethanol and acetic acid (3:1) for 24 hours. Root tips were macerated in 1N HCl for 10 minutes (Cold Hydrolysis) at room temperature. The slides were staining in 2% Fe-acetocarmin for 10 hours.

Data analyses

Morphological studies

For morphological studies 43 morphological characters including 16 qualitative and 26 quantitative characters were studied following the protocols of (Ashfaq *et al.* 2019; Attar *et al.* 2019; Gul *et al.* 2019a; Gul *et al.* 2019b; Kandemir *et al.* 2019; Shah *et al.* 2018a, 2018b; Zaman *et al.* 2019) (Table 2).

Table 2. List of selected characters and their codes in morphological studies.

No. Characters	Numerical code
1 Plant height	mm
2 Length of basal leaves	mm
3 Width of basal leaves	mm
4 Length of stem leaves	mm
5 Width of stem leaves	mm
6 Bract length	mm
7 Width bract	mm
8 Length pedicel	mm
9 Number of seeds per capsule	
10 Number of flowers per inflorescence	
11 Number of calyx	
12 Length calyx	mm
13 Width calyx	mm
14 Number of petal	
15 petal length	mm
16 Petal width	mm
17 Cleft size of petals	mm
18 Inter node length	mm
19 Number of stamen	
20 Number of stigma	
21 Capsule length	mm
22 Seed length	mm
23 Seed width	mm
24 Cleft size of capsule	mm
25 Number suture capsules	
26 Veins number sepals	
27 Growth period	0-annual 1- perennial
28 Bract apex	0-acute 1- narrow 2- absence
29 State of stem	0-unbranched 1- branched
30 State of stem strength	0-thin 1- strong
31 Hairs of stem	1-unilateral hair 2- multilateral hair
32 Cross-section of stem	0-round 1- rectangular 2- elliptical
33 Shape of basal leaves	0-linear 1- linear-lanceolate
34 Basal leaves apex	0-acute 1- narrow
35 Basal leaves petiole	0-absence 1- presence
36 Hair of basal leaves petiole	0-absence 1- presence
37 Shape cauline leaves	0- linear 1- linear-lanceolate
38 Cauline leaves apex	0- acute 1- narrow
39 Cauline leaves petiole	0-absence 1- presence
40 Hair of cauline leaves petiole	0-absence 1- presence
41 Hair of cauline leaves margin	0-absence 1- presence
42 Hair of cauline leaves lamina	0-absence 1- presence
43 Shape of bract	0-linear 1- linear-lanceolate

Morphological traits were standardized (Mean = 0, Variance = 1) and used to estimate Euclidean distance for ordination analyses (Podani 2000). PCA (Principal components analysis) biplot and MDS (Multidimensional scaling) were applied for grouping and identify the most variable morphological traits of among the populations (Podani 2000). We used from PAST version 2.17 (Hammer *et al.* 2012) for multivariate statistical analyses.

Molecular analyses

ISSR bands scored as present (1) or absent (0). Genetic polymorphism was determined by genetic diversity parameters: Shannon information index (I), percentage of polymorphism, the number of effective alleles and Nei's gene diversity (H) (Freeland *et al.* 2011). Neighbor-Net networking was used for Nei's genetic identity among studied populations (Huson and Bryant 2006; Weising *et al.* 2005). We used from PAST ver. 2.17 (Hammer *et al.* 2012), SplitsTree4 V4.13.1 (2013) and DARwin ver. 5 (2012) software for analysis data.

For AMOVA (Analysis of molecular variance) we used of GenAlex 6.4 software (Peakall and Smouse 2006; Meirmans and Van Tienderen 2004) that was determined Genetic differentiation of the species and Neis G_{st} analysis in GenoDive ver.2 (2013) (Hedrick 2005; Jost 2008) were used to revealed genetic distance of the species.

First data were scored as dominant markers (ISSR) so we used from STRUCTURE analysis for estimate the parameters that related to gene flow among studied population. Burn-in = 10000, and 10 runs were performed for relationship between Genetic structure and distance of geographical. Maximum likelihood method and Bayesian Information Criterion (BIC) was studied by structure analysis (Falush *et al.* 2007; Evanno *et al.* 2005; Meirmans 2012). Gene flow was determined by Calculating Nm from G_{st} by PopGene ver. 1.32 (1997). (Pritchard *et al.* 2000).

RESULTS

In this study 11 populations of *Stellaria media* were selected from northern regions of Iran. Genetic diversity parameters revealed that the highest percent of genetic polymorphism (48.89%) and gene diversity (0.179) exist in Guilan, Bandar Anzali, (population No.5), while the lowest amount of genetic polymorphism (13.33%) showed in population Guilan, Road side Bandar Anzali (No.8) Table 3.

Table 3. Genetic diversity parameters in the studied populations. (N = number of samples, Ne = number of effective alleles, I= Shannon's information index, He = gene diversity, UHe = unbiased gene diversity, P%= percentage of polymorphism, populations).

Pop	N	Na	Ne	I	He	uHe	%P
pop1	6.000	0.633	1.136	0.120	0.080	0.087	23.33%
pop2	8.000	0.644	1.119	0.121	0.077	0.083	25.56%
pop3	23.000	0.756	1.140	0.138	0.088	0.090	32.22%
pop4	5.000	0.511	1.123	0.109	0.073	0.081	20.00%
pop5	10.000	1.011	1.312	0.265	0.179	0.188	48.89%
pop6	6.000	0.944	1.279	0.231	0.158	0.172	40.00%
pop7	5.000	0.533	1.118	0.101	0.068	0.075	18.89%
pop8	4.000	0.422	1.099	0.078	0.054	0.061	13.33%
pop9	6.000	0.678	1.140	0.120	0.081	0.089	21.11%
pop10	6.000	0.922	1.260	0.227	0.152	0.166	42.22%
pop11	6.000	0.878	1.217	0.198	0.130	0.142	40.00%

AMOVA test showed that, 40% of total genetic diversity was within population and 60% was among population. Hedrick standardized fixation index makes of genetic distance among the studied populations. We have moderate level for AMOVA produced after 999 permutations (G'_{st} = 0.515, P = 0.001) and Hedrick differentiation index (D-est = 0.331, P = 0.001). Our results showed that the populations of *S. media* are differentiated from each other.

Populations genetic affinity

Neighbor-Net network and Nj tree revealed identity results but here only Neighbor-Net network is discussed (Figure 2). In the network showed that the populations 1 and 4, as well as populations 7 and 8 show are placed close to each other and they have closer genetic affinity. The populations 3 and 5, 6, 11 are differentiated from the other populations.

The studied specimen in MDS plot revealed that they were stay in different groups, which this results were in agreement with the AMOVA results (Figure 3). The relationship between altitude distance and genetic distance by Mantel test after 5000 permutations makes significant in these populations (r = 0.38, P = 0.001). We have isolation in *Stellaria media* occurred that we have low amount of gene flow due to geographically more distant of populations.

Populations genetic structure

The result carried out on STRUCTURE analyses by Evanno test which makes a peak at k = 9 (Figure 4). Fur-

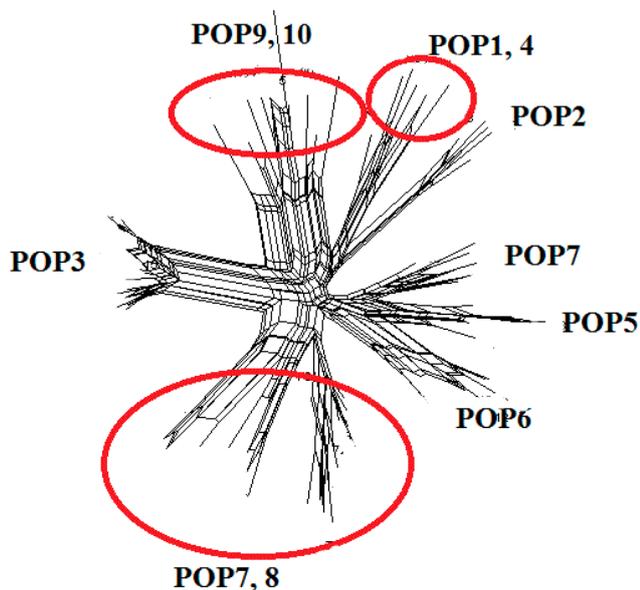


Figure 2. Neighbor-Net network of populations in *S. media* based on ISSR data.

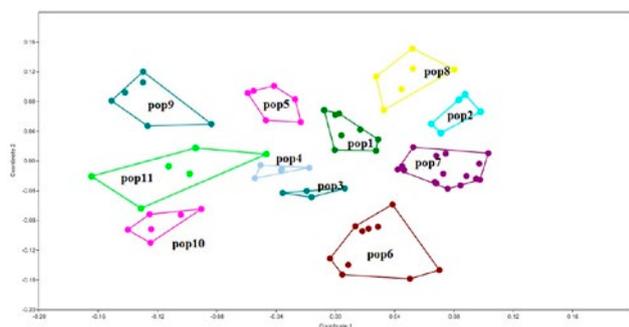


Figure 3. MDS plot of populations in *S. media* based on ISSR data.

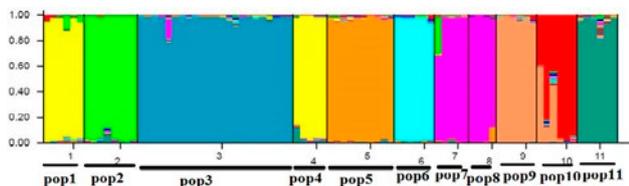


Figure 4. STRUCTURE plot of *S. media* populations based on $k = 9$ of ISSR data.

thermore, STRUCTURE analyses shown genetic identity between populations 1 and 4 (similarly colored), populations 7 and 8, like populations 9-10. But also it indicated genetic difference of populations 3 and 5 (differently colored), likes 6 and 11.

The results of Reticulogram (Figure 5), indicated some of shared alleles that is based on the least square

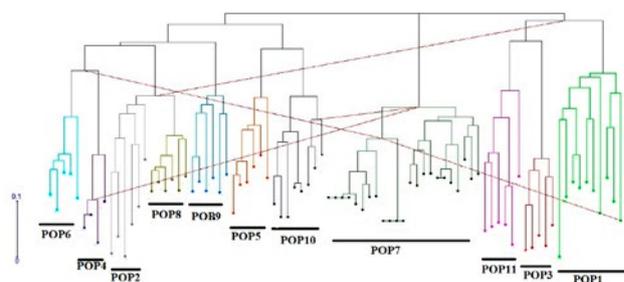


Figure 5. Reticulogram of *S. media* populations based on least square method analysis of ISSR data. (Population numbers are according to Table 1.

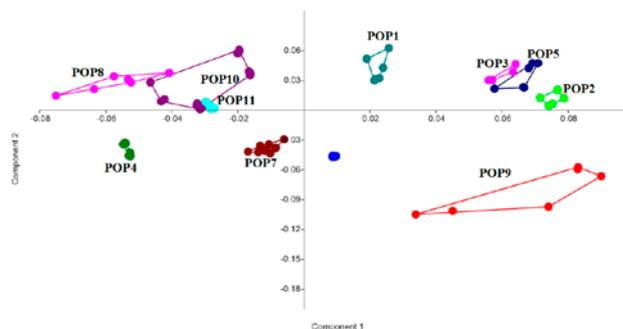


Figure 6. PCA plot of *S. media* populations based on morphological characters.

method among populations 10 and 4, 6 and between 7 and 4 and 10, also between 3, 11, 1 and 2 and 8. The mean $N_m = 0.29$ that is very low level of genetic diversity and supports genetic stratification as showed by STRUCTURE analyses and K-Means. N_m result agreed with population assignment test and cannot showed gene flow among these populations. In total ten ISSR primers produced 90 bands, fragment size ranged from 100 to 2800 bp.

Morphometric analyses

ANOVA test for 85 plant specimen were examined from 11 populations. Our results indicated significant difference in compare with the studied populations ($P < 0.05$). Ordination plot and other analyses produced similar result among populations (Figure 6). Our result revealed that among of the studied populations exist of morphological divergence and this divergence was due to quantitative traits. For example, length of stem leaves character separated population No. 9, but the populations 3 and 5 separated from the other populations due to character calyx length.

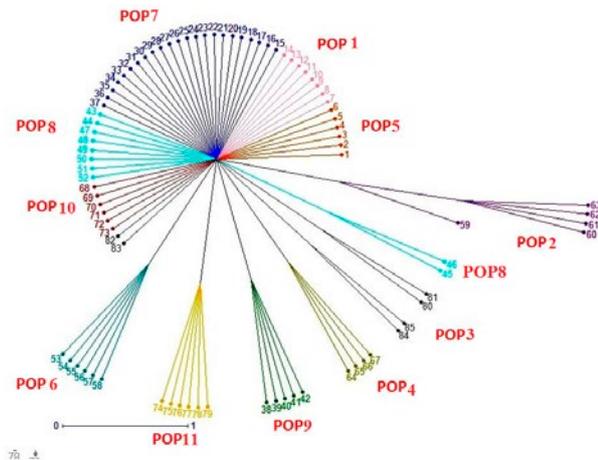


Figure 7. Consensus tree of morphological and molecular data in *S. media* populations.

We performed for both morphological and ISSR data a consensus tree (Figure 7). It indicated that some population are differenced from other population based on both morphological and molecular characters.

Karyological characteristics

In this study three populations of *S. media* show a tetraploid level, $2n=40$ (Figure 8a), six populations show a tetraploid level, $2n=42$ (Figure. 8b) and two populations show a tetraploid level, $2n=44$ (Figure. 8c) is in accordance with previous report (Morton 2005; Runemark 1996). There are high morphological variations in populations of *S. media* so that in some references subspecies have been defined for these taxa. The results show that such variations have chromosome number differences in Iran as most morphological variations were considered from different parts of Iran for this study.

In *S. media* have been reported $2n=28, 36, 40, 42$ and 44 from Eurasia with $2n=40$ predominating

(Federov 1974; Löve and Löve 1975; Moore 1973). This species shows a high phenotypic plasticity and genotypic flexibility.

DISCUSSION

According to Çalişkan (2012) genetic diversity provides information about adapt to changing environments, understanding of positive influence in the conservation of endangered species, hybridization and gene flow among the populations. This study evaluates on the use of Inter simple sequence repeats markers for compare gene flow and relationships within the population of *S. media* in Iran. Verkleij, *et al* (1980) showed that Amylases isoenzymes could be successfully applied to assess interpopulational variation in *Stellaria media*.

S. media has many medicinal properties and distributed in our country, however, we provided information on current taxonomic, molecular study and geographical distance. The present study indicated data about gene flow and genetic structure in some part of Iran. Chickweed can any time of the year at all germinate and flower. System pollination is mainly self-pollinating, but sometimes can occur cross-pollination by flies and insects.

According to Chater and Heywood (1993) *Stellaria media* widespread weedy species and it is the accepted name. There are three subspecies; 1- subsp. *media*, 2- subsp. *Cupaniana* and 3- subsp. *postii* but some people showed that subsp. *cupaniana* (Sinha 1965; Scholte 1978) and subsp. *postii* (Sinha 1965) should be included in *S. neglecta*. According to Fedorov (1969) chromosome numbers that have been reported for *S. media* included $2n = 24, 28, 36, 38, 40, 42$ and 44 from many parts of the world. However, chromosome numbers $2n = 40, 42$ and 44 are the most commonly reported and this species revealed a high degree of genotypic variation that is highly correlated with its reproductive (Freeland *et al.* 2011; Verkleij unpublished).

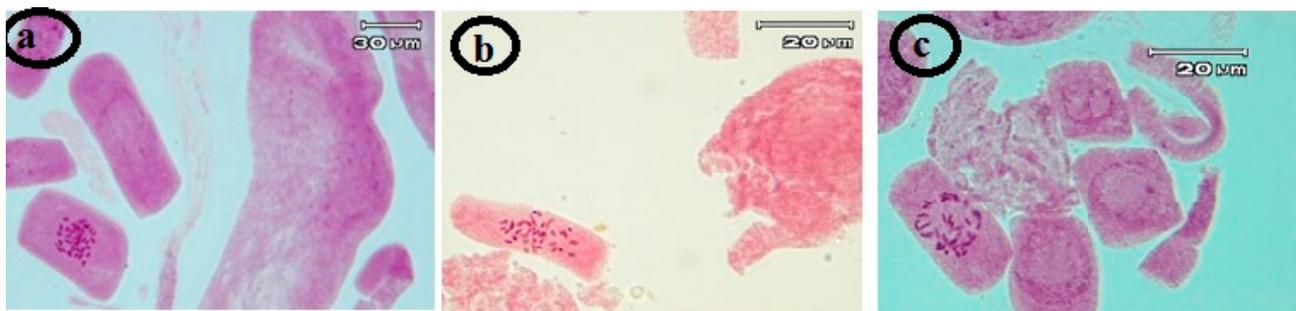


Figure 8. Micrographs of chromosomes of root tips in studied species. a) *S. media* ($2n=40$), b) *S. media* ($2n=42$), c) *S. media* ($2n=44$).

S. media is annual, characterized by the presence of five sepals and petals which are usually bifid; (Whitehead and Sinha 1967). Generally, within family Caryophyllaceae diversity of morphological features makes taxa complicated to be delineated and identified (Whitehead and Sinha 1967). *S. media* is occurring on abandoned fields and commonly sensitive to disturbance of its habitat. Between *S. pallida* and *S. media* there are crossing barrier and they are self-pollinating (Peterson 1936), this happened due to presence of polyploidy in *S. media* ($2n=40-44$) while observed the diploidy of *S. pallida* ($2n = 22$) (Scholte 1978; Slatkin 1993; Jolivet and Bernasconi 2007). Therefore, breeding systems plays role important in low level of gene flow in *S. media* (Hutchison and Templeton 1999; Medrano and Herrera 2008).

Our results provided that the seed morphologies of *Stellaria media* and *S. pallida* are similar. Seed coat cells are rounded polygonal and V-shaped margin. Based on these characters, we decided that *Stellaria media* could be differenced from *S. pallida*. Seed coat morphology observed of 18 species of *Stellaria* by Chen (2010). They stated that there are differences between *Myosoton* and *Stellaria*.

Rani *et al.* (2012) have studied some stem and leaf anatomical features through the pharmacognostical study for quality control of *Stellaria media*. Arora and Sharma (2012) did pharmacognostic and phytochemical studies of *Stellaria media* and showed the presence of epidermis, palisade cells, trichomes and vascular bundles in leaf.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

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