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Cytogenetic Diversity and Characterization of *Vicia sativa* Subspecies

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ABSTRACT

Background: *Vicia sativa* L. is variable genus comprised of several subspecies. Close relative species and subspecies of the cultivated species are easily usable gene sources because they have gained resistance against a wide range of biotic and abiotic stresses. The main objectives of this study are to identify and describe the cytogenetical and karyological characteristics of subspecies in the *Vicia sativa* complex.

Methods: The research material consisted of multiple entries collected from the five subspecies of nine taxa. All cytological observations made from root tips. Six chromosomal parameters (chromosome length, relative length, long arm length, short arm length, arm ratio and centromeric index) and five karyotype asymmetric parameters (difference in relative length, total form percentage, intrachromosomal asymmetry index, interchromosomal asymmetry index and mean centromeric asymmetry) were determined.

Result: It was determined that the chromosome number of subspecies were 2n =10-12. The haploid chromosome lengths of subspecies were 15.86-33.88 µm and the average chromosome lengths varied between 2.64-5.65 µm. According to the intrachromosomal karyotype asymmetry index analysis, subsp. *segetalis* was the most asymmetric karyotype and subsp. *sativa* "Antalya" was the most symmetric karyotype. According to the interchromosomal karyotype asymmetry index analysis, subsp. *angustifolia* was the most asymmetric karyotype and subsp. *nigra* was the most symmetric karyotype.

Key words: Asymmetry index, Chromosome, Karyotype, *Vicia sativa* subspecies.

INTRODUCTION

Vicia sativa L. is a genetically and phenotypically variable genus comprised of several subspecies. It is most commonly referred to as the *Vicia sativa* complex. Common vetch (*Vicia sativa* subsp. *sativa* L.) is the most widely grown subspecies used as a winter cover crop or green manure, although it has also been utilized for animal grazing, silage and hay (Seymour *et al*. 2002; Lahuta *et al.* 2020). The subsp. *cordata* and subsp. *macrocarpa* only have a limited agronomic value as fodder and the other subspecies of the complex have no agricultural value at all (Hanelt and Mettin, 1989; Van de Wouw *et al*. 2003). On the other hand, the close relative species and subspecies of the cultivated species are one of the gene sources used to develop superior varieties. This is because of gained resistance against a wide range of biotic and abiotic stresses.

The common consensus among studies concludes that this complex contains between 5 and 7 distinct subspecies, although there has been much debate on the topic. More specifically, the subspecies showed considerable variation in almost every trait, but, in particular, leaflet morphology, as well as basic chromosome number. Following this, they distinguished five main taxa at a subspecific rank. Most recently, Van de Wouw *et al*. (2001a,b) combined previous knowledge in a study and proposed five taxa of the *Vicia sativa* aggregate; subsp. *cordata*, subsp. *macrocarpa*, subsp. *nigra*, subsp. *sativa* and subsp. *segetalis*. Additionally, some aggregates within the complex have been considered to be distinct species by several authors and have subsequently been divided into further subspecies or varieties (Mettin and Hanelt, 1973; Potokina, 1997).

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Some cytogenetical and karyological studies have been conducted on the *Vicia sativa* complex up to date. Several experiments have indicated that there are three distinct chromosome numbers among the subspecies of *Vicia sativa*; 2n = 10, 12 and 14. To understand this, mixed populations of two, three, or four subspecies were observed in their natural habitats (Donnelly and Clark, 1962; Hollings and Stace, 1974; Ladizinsky and Temkin, 1978; Zohary and Plitmann, 1979; Ladizinsky, 1981; Celiktas *et al*. 2006). Some studies based on chromosome size, centromeric index and banding patterns have been conducted on *Vicia* species (Navratilova *et al*. 2003; El-Shanshoury, 2007). Controlled pollination experiments showed that *Vicia sativa* L. were automatic self-fertilizing species (Zhang and Mosjidis, 1995). Recently, Kartal *et al*. (2020) also concluded that successful interspecific hybridizations within the *Vicia sativa* complex were possible, but, nevertheless, infertilities were observed at generation F_1 and F_2.

In order to create novel morphological combinations through the hybridization of *Vicia sativa* aggregates and to enrich the gene pool for future breeding studies, individual chromosome types and karyotypes need to be investigated for all subspecies. The main objectives of this study are to identify and describe the cytogenetical and karyological characteristics of subspecies in the *Vicia sativa* complex.

MATERIALS AND METHODS

This research was carried out at the Ankara University Faculty of Agriculture, Biotechnology Laboratory between 2016-2017. The seeds of the accessions used in this research were kindly provided by Esvet Acikgoz (Uludag University, Bursa, Turkey). The research material consisted of multiple entries collected from the five subspecies given in Table 1.

The seeds of all accessions were germinated in petri dishes at room temperature (25°C) for two or three days until a root was obtained at a length of around 3 cm. All cytological observations were then made from samples collected from root tips at 2-3 days old. The root tips were pre-treated in 6% α-monobromonaphtalane at 4°C for 7-8 h and then fixed in glacial acetic acid for 30 minutes and transferred to 70% ethanol for long term storage. For analysis, the root tips were hydrolyzed with 1 N HCl for 12-18 minutes at room temperature (25°C). After hydrolyzing, the root tips were stained with 2% aceto-orcein in absolute darkness for 2.5 h. Finally, samples were crushed in 45% acetic acid, as described by Martin *et al*. (2018). The chromosomes were observed during the mitotic metaphase using an Olympus BX-51 (Olympus Corp., Japan) microscope. The chromosome number and karyotype measurements were recorded for at least ten cells per accession. The selected cells were photographed using the Olympus BX-51 camera at room temperature (25°C), at an 8000x magnification and used for the measurements. Six chromosomal parameters, chromosome length (c), relative length (RL), long arm length (L), short arm length (S), arm ratio (AR: L/S) and centromeric index (S/C), were measured using the Micro Measure 3.3 program (Reeves 2001). Ideograms were drawn based on the ratio of long arm length to short arm length. Karyotype formulas of all *Vicia sativa* genotypes were determined using the method described by Levan *et al*. (1964). Five parameters were used in order to estimate the karyotype asymmetry. The difference in relative length (DRL:MaxRL – MinRL) (Zarco 1986) and total form percentage (TF, 100 × ΣS/CL) were calculated (Huziwara 1962). The intrachromosomal asymmetry index A_1 = [1 – (Σ (short arm/long arm)/n)] and the interchromosomal asymmetry index; A_2 = [standard deviation (S)/mean length (X)] calculated by Zarco 1986. The mean centromeric asymmetry (M_{CA}) parameter was used to calculate the asymmetry of each chromosome (Peruzzi and Eroglu, 2013).

Table 1: Type, location and collector numbers of studied *Vicia sativa* subsp.

Subspecies	Type	Accession	Origin
Subsp. *angustifolia*	Wild	"VIC 408"	Turkey
Subsp. *cordata*	Wild	"VIC 701"	Egypt
Subsp. *macrocarpa*	Wild	"5283"	Syria
Subsp. *macrocarpa*	Landrace	Ericek village	Turkey
Subsp. *nigra*	Wild	"K-1"	Turkey
Subsp. *sativa* "Antalya"	Cultivar	Antalya	Turkey
Subsp. *sativa* "Beyaz"	Cultivar	Beyaz	Turkey
Subsp. *sativa* "Soner"	Cultivar	Soner	Turkey
Subsp. *segetalis*	Wild	VIC420	Ukraine

RESULTS AND DISCUSSION

In this study, the chromosome numbers and detailed chromosome measurements for the *Vicia sativa* subspecies were determined. All analyzed accessions of the *Vicia sativa* complex were diploid. The subsp. *angustifolia*, *macrocarpa, sativa* and *segetalis* were found to have 2n=2x=12 chromosomes (Fig 1 and 2), whereas the subsp. *cordata* and *nigra* were found to have 2n = 2x = 10 (Fig 1 and 2).

The results also confirmed that the basic chromosome numbers were x = 5 and 6, as had been previously reported. Additionally, the karyotype formulas of *Vicia sativa* subspecies with the same basic chromosome numbers were found to have higher similarity rates. Ideograms (Fig 2) were generated to using the mean values of chromosomes. Karyotype formulas and quantitative analyses of the *Vicia sativa* subspecies differed among cultivars as well as subspecies of different origins. However, the karyotypical differences were found to be much higher among subspecies of different origins. The chromosomes observed in the study were mainly submetacentric or subtelocentric types.

Cv. Antalya and subsp. *macrocarpa* "Ericek" were shown to have the same karyotype formula of 2n = 12 = 6m + 6sm. Following this, subsp. *angustifolia* (2 m + 2 sm + 8 st) and cv. Soner (4 sm + 8st) karyotypes had four pairs of subtelocentric chromosomes. It was determined that four pairs of chromosomes were also submetacentric in cv. Beyaz (4 m + 8 sm) and subsp. *macrosperma* "5283" (8 sm + 4 st). The karyotype formulas of subsp. *cordata* and subsp. *nigra,* with the chromosome number 2n = 10, were determined to be 2 sm + 8 st and 2 m + 4 sm + 4 st, respectively (Table 2).

When the average long arm length of *Vicia sativa* subspecies was analyzed, subsp. *segetalis* was observed to be the longest. It was followed by subsp. *cordata* and subsp. *angustifolia*. It was also determined that cv. Antalya had the shortest average long arm length among the subspecies. Additionally, chromosome no 1 of subsp. *segetalis* had the highest long arm length (7.038 µm) out of all the genotypes (Table 3). The average chromosome lengths of the accessions varied between 2.64 - 5.65 µm, as indicated in Table 2. When the length of the longest chromosome of the accessions was compared, the range was found to be 3.251 µm and 9.036 µm in Antalya and subsp. *angustifolia*, respectively. On the other hand, the shortest chromosome length range was also observed in cv. Antalya with 1.686 µm and subsp. *cordata* with 4.477 µm (Table 3).

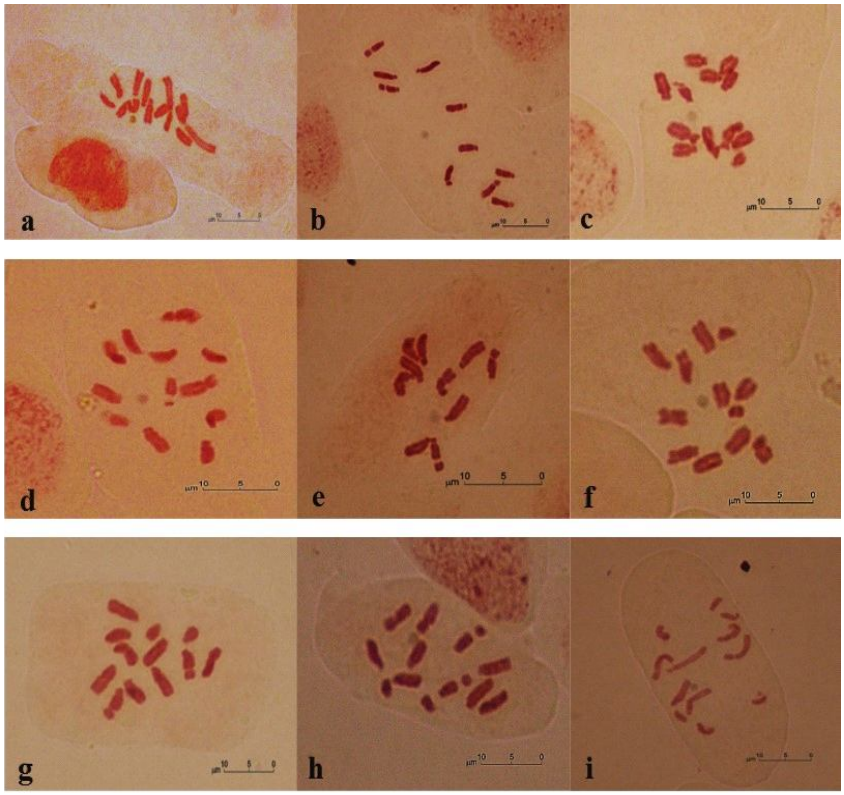


Fig 1: Somatic chromosomes at mitotic metaphase of *Vicia sativa* subspecies.
(a: Subsp. *angustifolia* "VIC 408" b: Subsp. *cordata* "VIC 701", c: Subsp. *macrocarpa* "5283", d: Subsp. *macrocarpa* "Ericek", e: Subsp. *nigra* "K-1", f: Cv. Antalya, g: Cv. Beyaz, h: Cv. Soner and i: Subsp. *segetalis* "VIC 420").

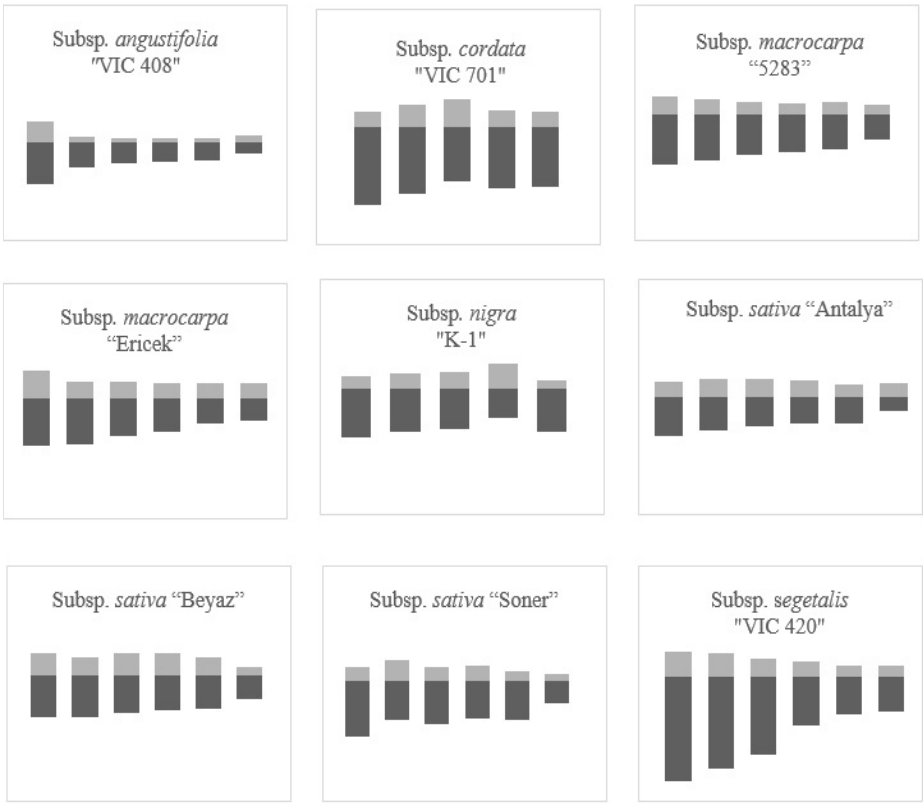


Fig 2: Ideograms of *Vicia sativa* subspecies.

Table 2: Karyotype characteristics of *Vicia sativa* subspecies.

Subspecies	2n	Karyotype Formula (2n)	S (µm)	L (µm)	C (µm)	R	CI	CL (µm)
Subsp. *angustifolia* "VIC 408"	12	2 m + 2 sm + 8 st	1.18 ± 0.38	3.26 ±0.61	4.44 ± 0.94	3.29 ± 0.52	0.26 ± 0.04	26.639
Subsp. *cordata* "VIC 701"	10	2 sm + 8 st	1.12 ± 0.14	3.85 ± 0.24	4.97 ± 0.20	3.67 ± 0.53	0.20 ± 0.03	24.871
Subsp. *macrocarpa* "5283"	12	8 sm + 4 st	0.95 ± 0.09	2.70 ± 0.25	3.66 ± 0.33	2.83 ± 0.12	0.26 ± 0.01	21.934
Subsp. *macrocarpa* "Ericek"	12	6 m + 6 sm	1.17 ± 0.14	2.36 ± 0.27	3.52 ± 0.38	2.05 ± 0.20	0.34 ± 0.02	21.145
Subsp. *nigra* "K-1"	10	2 m + 4 sm + 4 st	0.95 ± 0.17	2.56 ± 0.21	3.52 ± 0.10	3.18 ± 0.70	0.23 ± 0.05	17.585
Subsp. *sativa* "Antalya"	12	6 m + 6 sm	0.94 ± 0.05	1.70 ± 0.20	2.64 ± 0.23	1.81 ± 0.20	0.37 ± 0.03	15.863
Subsp. *sativa* "Beyaz"	12	4 m + 8 sm	1.41 ± 0.16	2.62 ± 0.20	4.03 ± 0.34	1.93 ± 0.16	0.35 ± 0.02	24.204
Subsp. *sativa* "Soner"	12	4 sm + 8st	0.94 ± 0.13	2.76 ± 0.30	3.70 ± 0.38	3.10 ± 0.35	0.25 ± 0.02	22.208
Subsp. *segetalis* "VIC 420"	12	2 sm + 10 st	1.20 ± 0.16	4.45 ± 0.81	5.65 ± 0.97	3.59 ± 0.22	0.22 ± 0.01	33.882

Abbreviations: S- Short arm length; L- Long arm length; C- Chromosome length; R- Arm ratio (long arm/short arm); CI- Centromeric index coefficient; CL- Total haploid chromosome length.

Table 3: Karyomorphological parameters of *Vicia sativa* subspecies.

Number	Type	Chromosome length (µm)	Relative length (%)	Long arm length (µm)	Short arm length (µm)	Arm ratio	Centromeric index
			subsp. *angustifolia* "VIC 408"				
I	sm	9.036	33.920	5.993	3.043	1.965	0.338
II	st	4.335	16.270	3.525	0.811	4.352	0.187
III	st	3.775	14.170	3.089	0.686	4.79	0.181
IV	st	3.453	12.960	2.751	0.703	4.258	0.203
V	st	3.311	12.430	2.588	0.723	3.891	0.219
VI	m	2.728	10.240	1.615	1.114	1.527	0.401
			subsp. *cordata* "VIC 701"				
I	st	5.595	22.500	4.699	0.896	5.246	0.160
II	st	5.239	21.060	3.984	1.255	3.185	0.239
III	sm	4.884	19.640	3.269	1.614	2.027	0.331
IV	st	4.677	18.800	3.719	0.957	3.940	0.205
V	st	4.477	18.000	3.583	0.894	4.019	0.200
			subsp. *macrocarpa* "5283"				
I	sm	4.797	21.870	3.466	1.331	2.607	0.277
II	sm	4.241	19.340	3.180	1.061	2.996	0.250
III	st	3.645	16.620	2.760	0.885	3.120	0.243
IV	st	3.461	15.780	2.621	0.840	3.254	0.242
V	sm	3.347	15.260	2.460	0.887	2.790	0.265
VI	sm	2.443	11.140	1.723	0.720	2.396	0.294
			subsp. *macrocarpa* "Ericek"				
I	m	4.985	23.580	3.122	1.863	1.670	0.375
II	sm	4.154	19.640	3.018	1.135	2.663	0.273
III	sm	3.593	16.990	2.542	1.051	2.419	0.293
IV	sm	3.212	15.190	2.255	0.957	2.356	0.300
V	m	2.656	12.560	1.672	0.984	1.795	0.372
VI	m	2.545	12.040	1.533	1.012	1.523	0.398
			subsp. *nigra* "K-1"				
I	st	3.784	21.520	3.049	0.735	4.148	0.194
II	sm	3.696	21.020	2.721	0.974	2.936	0.264
III	sm	3.511	19.970	2.511	1.000	2.616	0.286
IV	m	3.354	19.080	1.816	1.538	1.190	0.459
V	st	3.239	18.420	2.720	0.519	5.268	0.160

Table 3: Continue...

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				subsp. *sativa* "Antalya"			
I	sm	3.251	20.490	2.325	0.926	2.511	0.285
II	sm	3.067	19.330	2.017	1.05	1.928	0.342
III	m	2.836	17.880	1.771	1.065	1.673	0.375
IV	m	2.651	16.710	1.622	1.029	1.588	0.387
V	sm	2.372	14.950	1.614	0.758	2.138	0.319
VI	m	1.687	10.630	0.877	0.809	1.087	0.480
				subsp. *sativa* "Beyaz"			
I	sm	4.768	19.700	3.091	1.678	1.919	0.373
II	sm	4.473	18.480	3.091	1.382	2.242	0.309
III	m	4.421	18.260	2.737	1.684	1.631	0.381
IV	m	4.227	17.460	2.536	1.691	1.597	0.397
V	sm	3.850	15.910	2.501	1.350	1.869	0.351
VI	sm	2.465	10.180	1.771	0.694	2.558	0.282
				subsp. *sativa* "Soner"			
I	st	4.882	21.980	3.897	0.985	4.068	0.202
II	sm	4.174	18.800	2.745	1.430	1.933	0.343
III	st	3.955	17.810	2.975	0.980	3.545	0.245
IV	sm	3.727	16.780	2.623	1.104	2.636	0.295
V	st	3.371	15.180	2.713	0.658	4.154	0.195
VI	st	2.099	9.450	1.601	0.498	3.219	0.238
				subsp. *segetalis* "VIC 420"			
I	st	8.732	25.770	7.038	1.694	4.480	0.196
II	st	7.797	23.010	6.205	1.592	4.390	0.204
III	st	6.520	19.240	5.258	1.261	4.170	0.193
IV	st	4.383	12.940	3.313	1.070	3.472	0.246
V	st	3.322	9.810	2.535	0.787	3.241	0.236
VI	sm	3.128	9.230	2.342	0.786	2.990	0.251

Abbreviations: S- Short arm length; L- Long arm length; C- Chromosome length; R- Arm ratio (long arm/short arm); CI- Centromeric index coefficient; CL- Total haploid chromosome length.

The range of the total haploid chromosome length in the accessions was determined to be between 15.863 µm (cv. Antalya) and 33.882 µm (subsp. *segetalis*). Furthermore, cv. Soner (22.208 µm) and subsp. *macrosperma* "5283" (21.934 µm) are the genotypes with the most similar haploid chromosome lengths. This information has given insight into the chromatin content of the analyzed genotypes (Konischenko *et al*. 2014).

The results of the karyotype asymmetry index analysis showed that intrachromosomal asymmetry was higher than interchromosomal asymmetry in the karyotypes of *Vicia sativa* subspecies (Table 4). Subsp. *segetalis* had the highest A_1 (0.72) and M_{CA} (55.87) value and the lowest TF value (21.22%). Thus, subsp. *segetalis* was determined to be the most asymmetric karyotype. On the contrary, cv. Antalya was the most symmetric karyotype among the genotypes with A_1 (0.41) and M_{CA} (26.99) values and with the highest TF (35.54%) value. When the interchromosomal asymmetry results were analyzed (Table 4), subsp. *angustifolia* was shown to have the highest A_2 (0.52) and DRL (23.68) values and it was therefore determined to be the most asymmetric karyotype among the genotypes. Subsp. *nigra* had the lowest A_2 (0.06) and DRL (3.10) values.

In close agreement with this study, the same chromosome numbers were reported in earlier studies for the *Vicia sativa* subspecies analyzed in this paper. However, the subspecies may contain different chromosome numbers within itself. For example, the accessions of subsp. *sativa* showed 2n= 10, 12 and 14, subsp. *angustifolia*, *cordata* and *nigra* showed 2n= 10 and 12 and subsp. *macrocarpa* showed 2n= 12 and 14, in the mixed *Vicia sativa* populations (Yamamoto 1968; Hollings and Stace, 1974; Ladizinsky 1978; Ladizinsky and Temkin, 1978; Zohary and Plitmann, 1979; Kamari *et al*., 1994; Weber and Schifino-Wittmann, 1999; Arslan *et al*. 2012; Martin *et al*. 2018).

Analyzing karyotype asymmetry provides a good point of reference for the general morphology of the plant karyotypes (Zarco 1986; Zuo and Yuan 2011). Asymmetry is defined by the dominance of almost the same size m and sm chromosomes (Zuo and Yuan 2011; El-Bok *et al*. 2014). Changes in chromosome morphology have often been associated with evolution in higher plants. In this study, in order to determine the asymmetric values in the karyotypes of genotypes, the intrachromosomal (A_1) and interchromosomal (A_2) asymmetric indexes of Zarco (1986) were used. The intrachromosomal asymmetry index (A_1)

Table 4: Mean comparison of karyotype asymmetric parameters on different *Vicia sativa* subspecies.

Subspecies	DRL	TF (%)	A_1	A_2	M_{CA}
Subsp. *angustifolia* "VIC 408"	23.68	26.58	0.64	0.52	48.81
Subsp. *cordata* "VIC 701"	4.50	22.58	0.70	0.09	54.62
Subsp. *macrocarpa* "5283"	10.73	26.10	0.64	0.22	47.57
Subsp. *macrocarpa* "Ericek"	11.54	33.11	0.49	0.26	33.15
Subsp. *nigra* "K-1"	3.10	27.10	0.59	0.06	45.54
Subsp. *sativa* "Antalya"	9.86	35.54	0.41	0.21	26.99
Subsp. *sativa* "Beyaz"	9.52	35.03	0.47	0.20	30.87
Subsp. *sativa* "Soner"	12.53	25.46	0.65	0.25	49.31
Subsp. *segetalis* "VIC 420"	16.54	21.22	0.72	0.42	55.87

Abbreviations: DRL- Difference of relative length; TF- Total form percentage; A_1- Intrachromosomal asymmetry index; A_2-Interchromosomal asymmetry index; M_{CA} - Mean centromeric asymmetry.

indicated the arm ratio of each pair of homologous chromosomes, where the interchromosomal asymmetry index (A_2) was applied to variation coefficient. The most asymmetric values in the genotypes were therefore determined by the indices A_1 and A_2.

CONCLUSION

Theoretically, differences observed in length and the structure of chromosome morphology could be explained away as gradual alterations, which occurred through the evolution of the karyotype during natural or manual selection.

Detailed chromosome measurements and karyotype asymmetry degrees of all *Vicia sativa* subspecies are not available in the published literature. However, throughout this experiment, the cytological and karyological characteristics of *Vicia sativa* subspecies have been identified. Our findings could play a significant role in gaining an understanding of their genetic stability and potential for utilization in breeding programs. The gene pool that can be used in plant breeding programs is divided into three groups, according to hybridizability and proximity relationships (Harlan and de Wet, 1971; Von Bothmer *et al*. 2003; Kanwar *et al*. 2020). The primary gene pool group consists of modern (elite) varieties, old varieties, landraces and close relative species. Subspecies, which are compatible and similar in terms of cytological characteristics, are more likely to succeed in the transfer of desirable features during cross-breeding and/or self-fertilization studies (Devi *et al.* 2020). Chromosome numbers are 2n=2x=10, subsp. *cordata* and subsp. *nigra* to each other, are thought to give fertile progeny when crossed obtained. It can be speculated that fertile offspring can be obtained from the hybrids of other subspecies and cultivars with a chromosome number of 2n=2x=12. Research has accelerated putting superior features from close species and subspecies into cultivated types with the techniques and technologies developed in recent years. In this study, the potential of the hybridization of subspecies with cultivated types and possible genetic barriers among subspecies have been revealed at the cytological level.

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