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Characterization of the Genetic Diversity of Some Species of Genus *Vicia* Using ISSR and ITS Molecular Techniques

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THE HIGH economic importance of the genus *Vicia* has led to a large body of studies on the molecular characterization and investigation of phylogenetic relationships among species that belonged to this genus. The Mediterranean area is the principal center of distribution and diversification where polymorphism has been associated with the geographical origin of germplasm (ICARDA, 2013). The extent of genetic diversity in germplasm can be assessed through morphological characterization and genetic markers. The molecular techniques have a more accurate evaluation of the genetic and environmental components of variation, bringing greater precision to measures of genetic diversity. The present study is a molecular analysis of *Vicia* germplasm through ISSR and ITS techniques. The objectives were to determine the genetic diversity and phylogenetic relationship among defined 19 accessions, collected by ICARDA from different countries and habitats, representing nine *Vicia* species and subspecies: *V. ervilia*; *V. monantha; V. villosa* subsp. *villosa*, *V. villosa* subsp. *dasycarpa* and *V. villosa* subsp. *eriocarpa; V. sativa* subsp. *nigra*, subsp. *amphicarpa*, subsp. *macrocacrpa* and subsp. *sativa*. The patterns of variation of those accessions were studied by the numerical analysis of data to generate pertinent genetic information for the complement of passport data of ICARDA germplasm collections.

Keywords: Genetic diversity, ISSR, ITS Phylogeny, *Vicia.*

Introduction

Vicia L. is commonly found throughout Europe, North and South America, and the Mediterranean region (Weber & Wittmann, 1999; Naranjo et al., 1998). The Mediterranean region has been identified as its primary distribution and diversification hub, where polymorphism has been linked to its geographical origin (Van de Wouw et al., 2001).

This genus includes extensively cultivated *Vicia faba*, *Vicia narbonensis*, *Vicia sativa* subspecies *sativa* (common vetch), *Vicia hyaeniscyamus*, *Vicia noeana*, and *Vicia sativa* (Maxted et al., 1991; Maxted, 1995). *Vicia sativa* L., *V. villosa Roth*, and *V. narbonensis* L. are primarily planted for their high-quality fodder and protein-rich seeds (28 to 32%) in Spain, Turkey, Jordan, Syria, and Iraq (Siddique et al., 1996; Caballero et al., 2001). *Vicia sativa* subsp. *sativa* (vetch) is a common winter crop utilized as grazing, silage, and hay. (Sullivan, 2003).

Previously, morphological or cytological approaches have been utilized to assess the genetic variability of commercial crops (Islam & Shepherd, 1991). However, these techniques, while deemed successful in a number of cases, are not suitable for large-scale screening as these are time-consuming and use a limited number of markers. Molecular techniques may provide a simple, low cost solution for large scale screening needs.

Molecular techniques with an emphasis on nucleic acids have been commonly used to investigate genetic diversity. The inter-simple sequence repeats (ISSR) technique has been widely used in genetic diversity studies, genetic

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evolution and systematic specification, and gene mapping (Paplauskienė & Dabkevičienė, 2012). The ISSR technique is known to be sensitive enough to detect low levels of genetic diversity while still having the benefits of ease and low cost. It can be used to detect the genetic diversity in Vicia, chickpea, Katsouni pea and Stylosanthes scabra (Sarwat, 2012; Singh et al., 2014; Laureles et al., 2015; Rajkovic′ et al., 2015; Costa et al., 2019; Stavridou et al., 2020)

For more exact conclusions, DNA sequencing can be used as sequences are deemed to be largely stable throughout evolution and speciation. One sequence, that is, the internal transcribed spacer (ITS) region of ribosomal DNA, has provided promising results for phylogenetic analyses (Mishra et al., 2016). Most eukaryotes have hundreds of copies of the ITS region, which includes the 18S, ITS1, 5.8S, ITS2, and 28S regions (Wang et al., 2011). In a study by Han et al. (2021), they were able to discriminate between Korean *Vicia* species using a molecular phylogenetic analysis of the ITS2 region with two bar code regions.

In this study we aim to determine the genetic diversity and phylogenetic relationship among 19 defined accessions representing 4 *Vicia* species and 9 subspecies from International Center for Agricultural Research In the Dry Areas (ICARDA, 2003) collection. These species and subspecies were from different countries in the Middle East and represent pertinent genetic information for the passport data complement of ICARDA germplasm collections.

Materials and Methods

Seeds collection

The 19 accessions used in this current study were collected from different geographic origins representing 4 *Vicia* species and 9 subspecies, which were chosen for ISSR analysis. This choice has considered the principal coordinate analysis carried out in a previous study conducted by El-Badan et al. (2021) on 51 accessions using SDS-PAGE protein analysis. These 19 accessions were found to be either far from its groups or intermingled with other groups which represent the following sections: Vicia (*V. sativa* L.; accessions of subspecies *macrocarpa* intermingled with accessions of subspecies *amphicarpa* – accessions of subspecies *nigra* intermingled with that of subspecies *sativa*), Cracca (*V. monantha* Retz, *V. villosa* Roth; accessions of subspecies eriocarpa were deviated than other subspecies *dassycarpa* or subspecies *villosa*), and Ervum (accessions of *V. ervilia* L. have deviated from their association). The accession passport information of the examined species is listed in Table 1.

Genomic DNA extraction and ISSR fingerprinting

DNA was extracted from 500 mg dry seed samples using the modified cetyltrimethylammonium bromide (CTAB) method (Doyle & Doyle, 1990). DNA was kept in 25–30µL distilled water and preserved below 20˚C until used. Its quality was evaluated using 1% agarose gel electrophoresis.

Six of ten ISSR primers with a GC content of 50% –73% were able to generate clear scorable bands with good, reproducibility and amplification patterns (Table 2). These primers were 9–16 mers with various di- or trinucleotide repeats. The ISSR technique was performed following Reddy et al. (2002). All amplifications were carried out according to Sarwat (2012), using PCR MyGene series Peltier, LongGene thermal cycler, USA. Amplified PCR products were electrophoresed in 2% agarose gel containing 0.5 µl of ethidium bromide and photographed via a gel documentation system. A 1 kl base pair DNA ladder (Thermo Fisher Scientific Co.) was used as a marker.

Genomic DNA isolation for ITS analysis

The total cellular DNA was extracted from seeds of ten accessions (one accession each from *V. ervilia*, *V. monantha*, subspecies *nigra*, subspecies amphicarpa, subspecies macrocarpa, subspecies eriocarpa, subspecies dassycarpa, and subspecies villosa and two accessions from subspecies *sativa*) by GeneJet Genomic DNA Purification Mini Kits (Thermo Scientific) following the manufacturer's protocol. Amplifications of ITS regions were carried out using ITS1 and ITS4 primers. The sequence of ITS1 primer is 5`TCC GTA GGT GAA CCTGCG C 3`; it then binds to the end of 18S nrDNA gene. While ITS4 is 5` TCC TCCGCT TAT TGA TAT GC 3`; it then binds to the end of the 28S nrDNA gene. The reaction mix contained 1µL DNA, 1µL of each primer and 25µL master mix (DreamTaq PCR Master Mix, fermentase); it was then filled to 50µL with DNase-free water. The thermal cycler was set to run at 95°C for 5min for initial denaturation; there after we ran 34 cycles at 95°C for 1min , annealing at 58°C for 1min, extension at 72°C for 1min and 10min for

final extension (Shiran et al., 2014). Then, 2µL of each PCR product and DNA ladder (fermentase) were loaded on 2% (w/v) agarose gel, stained with ethidium bromide, separated by electrophoresis (80 V, 150mA), and viewed on UV plate for photographing. The Qiagen PCR Cleanup Kit was used for DNA purification before sequencing the PCR products by Sigma Co. through Macrogen Inc. Seoul, South Korea.

TABLE 1. Passport information of the 19 *Vicia* investigated accessions from ICARDA. m.asl*:* meter above sea level, IG: ICARDA catalogue number

Vicia species		Accessions	Origin country	Province	Crop_nr (IFVI)	IG	Longitude°	Latitude (N)	Alt m.asl
V. ervilia L.		e 4	LBN	Baalbek	2698	62647	E36° 04′	N34° 02′	100
		e 7	JOR	Tafila	4244	64193	E35° 35′	N30° 54′	900
V. villosa Roth	subsp. *dasycarpa*	v.d 3	TUR	Antalya	4059	64008	E31° 11′	N36° 56′	90
	subsp. *villosa*	v.v 1	SYR	Hama	3372	63321	E36° 19′ 20′′	N35° 54′ 30′′	720
	subsp. *eriocarpa*	v.e	SYR	Al Hasakah	683	66320	E41° 14′ 26′′	N37° 02′ 57′′	464
*V. monanth***a** Retz		m 2	SYR	Sweida	5129	108430	E36° 34′ 30′′	N32° 49′ 50′′	920
		m 4	EGY	Marsa Matrouh	4262	64211	E28° 20′	N31° 03′	30
		m 9	MOR	Marrakech	4883	107589	W07° 05′	N31° 47′	765
V. sativa L.	subsp. *nigra*	s.n 1	TUR	IZMIR	1416	61365	E27° 39′	N38° 24′	210
		s.n 3	EGY	Al Buhayrah	4215	64164	E30° 02′	N31° 09′	~6
		s.n 5	EGY	Alexandria	4268	64217	E29° 57′	N31° 10′	10
	subsp. *Amphicarpa*	s.am2	SYR	Sweida	5132	108433	E36° 43′ 00′′	N32° 52′ 00′′	1106
	subsp. *Macrocarpa*	s.mac 1	TUR	Aydin	4144	64093	E27° 21′	N37° 45′	200
		s.mac 4	MOR	Tetouan	4595	64544	W05° 32′	N35° 33′	230
	subsp. *sativa*	s.s 2	TUR	Yozgat	3610	63559	E34° 56′	N39° 51′	1300
		s.s 4	IRN	Sharekod	455	60404	E50° 52′	N32° 20′	2070
		s.s 6	JOR	Balqa	4317	64266	E35° 43′	N32° 06′	760
		s.s 8	CYP	Nicosia	708	60657	E33° 21′	N35° 09′	~134
		s.s 14	MOR	Marrakech	4362	64311	W07° 45′	N31° 20′	880

TABLE 2. Primers sequence used in the ISSR-PCR technique

Primers	Primer sequence 5'→3'	Repeat motif	GC (%)	T_m(°C)
ISSR-1	CA CA CA CA CA CA GG	$(CA)_6$	57	39
ISSR-3	GT GT GT GT GT GT GG	$(GT)_6$	57	39
ISSR-4	GA GA GA GA GA GA CC	$(GA)_6$	57	39
ISSR-6	CAC CAC CAC GC	$(CAC)_3$	73	33
ISSR-9	CA CA CA CA CA CA AG	$(CA)_6$	50	37
ISSR-10	CT CT CT CT CT CT CT CT GC	$(CT)_8$	56	51

Data scoring and assessment of total genetic diversity

The TotalLab image analysis software (version 1.1.4301, 26877) was used to analyze the digital images of ISSR gels and to further determine the band presence and absence. The polymorphic band percentage (Pb %) was calculated according to Liengsiri et al. (1990). The total genetic diversity was estimated through several descriptive measures of diversity summarized in International Plant Genetic Recourses Institute (IPGRI, 1993). The genetic diversity statistics were generated using POPGen software version 1.32 (Yeh et al., 1999) to describe the genetic variation of inter- and intrapopulation levels as:

i. The observed number of alleles (na) and the effective number of alleles (ne), (Kimura & Crow, 1964).

ii. Nei' s gene diversity (Nei, 1978), **($H = H_T - H_s$)**, where H_T is the total gene diversity and H_s is the genetic diversity within the population.

iii. Shannon's information index (**I**) (Lewontin, 1972), **($I = -\sum_{Pi} Log2_{Pi}$),** where "$_{Pi}$" is the gene frequency on locus "$_i$".

iv. Coefficient for gene divergence (G_{st}), **($G_{st} = (H_T - H_s) / H_T$**.

The efficiency of ISSR primers was assessed using four indices, that is polymorphic information content (**PIC**), effective multiplex ratio (**EMR**), resolution power (**RP**), and marker index (**MI**) (Mohanty et al., 2010). PIC is considered as average heterozygosity and calculated as ∑ (1-Pi2)/ n, where Pi is i^{th} allele frequency (band presence) and 1-Pi is the null allele frequency (Weir, 1990). EMR is the product of the fraction and the number of polymorphic bands (Powell et al., 1996). Increasing EMR value, correlates with increasing marker performance in terms of detecting polymorphisms (Sarwat, 2012). Rp is the total band informativeness (Ib) which equals 1- (2 x (0.5-p)) where "p" is the proportion of genotypes containing "I" band (Prevost & Wilkinson, 1999). MI is often to characterize the ability of each primer to detect polymorphic loci among genotypes and is defined as the product of the percentage of polymorphism percent and PIC according to Sorkheh et al. (2007).

The ITS region of the nrDNA gene of the ten *Vicia* accessions utilized in this study was sequenced using a Macrogen Ltd ABI automated sequencer (Korea). The acquired sequence chromatograms were edited using the BioEdit Sequence Alignment Editor software (Hall, 1999). The CLUSTAL X2 program version 2.1 (Thompson et al., 1997) was used to align these sequences with the published outgroup sequences, with default settings of 0.66 fixed gap penalty and 0.5 DNA transition weight.

To generate the best model, the phylogenetic tree was constructed using the maximum parsimony (MP) approach with PAUP version 4 and MEGA 4 software. The aligned sequences were then used to determine the number of substitutions per site using the following seven distance matrices: Kimura's two-parameter (Kimura, 1980), p-distance, Jukes & Cantor (1969), Tajima & Nei (1984), Tamura & Nei (1993), Tamura 3 parameter, and maximum composite likelihood estimation (Swofford, 2002). MEGA software version 4 (Tamura et al., 2007) was used to analyze the phylogenetic and molecular phylogenetic relationships using all these matrices and the four possible algorithms: neighbor-joining, unweighted pair group method with arithmetic mean, maximum parsimony, and minimum evolution. By bootstrapping the data with 1000 repeats and removing the gaps, branch reliability was evaluated.

The analysis was carried out with and without outgroups by choosing one *Lens* and four *Lathyrus* species (from GenBank/NCBI) identify the relationships among the ten *Vicia* accessions and determine the linkages that remain constant regardless of the outgroup. The ITS regions sequence data of the outgroup species were adopted from GenBank /NCBI: *Lens culinaris* (EU224443), *Lathyrus laxiflorus* (AY839367), *Lathyrus pratensis* (AY839384), *Lathyrus clymenum* (AY839346), and *Lathyrus spathulatus* (AY839392).

Aligned sequences were converted to the NEXUS format and analyzed by the Wagner parsimony method using the "branch- and – bound" and "heuristics" options of **PAUP** 4.0 (Swofford, 2002). Gaps were treated as missing data. The weighting scheme for MP analyses was determined by transitions/transversions ratios. Given the large data set, the heuristic

search used the tree bisection reconstruction (TBR) option with aAccelerated transformation character state optimization (ACCTRAN). Two indices were used to estimate the amount of phylogenetic information in the parsimony analysis for tree construction: consistency (**CI**) and homoplasy (**HI**) (Farris, 1989). Here, **CI**: is a measure of the parsimony fit of data to the resultant tree topology to assess the strength of the phylogenetic signal as a gage of confidence. It varies from 1 (perfect fit) to a value 0 (poorest fit). **HI** is **1-CI.** Uninformative characters can in-flat the CI, giving a result that indicates less homoplasy than is truly present. The distance matrices were calculated, and the g1 statistics were computed, using PAUP' s Random trees option.

Results

The detailed data measurement for each primer of the six used primers is attached in the Appendix. ISSR-PCR technique showed the variable size and number banding patterns with reproducible polymorphic bands, depending on their simple sequence repeat motifs of the accession (Fig. 1, Table 3). The total number of scorable bands were ranged from 25 to 36 bands with polymorphism percentage oscillated from 7% to 48%. One common band (about 62bp) was amplified from genomic DNA of the 19 accessions with ISSR-3 primer. Primers ISSR-4 and ISSR-6 amplified the highest number of unique bands (8), while primers ISSR-3 and ISSR-9 amplified only two unique bands (Table 3).

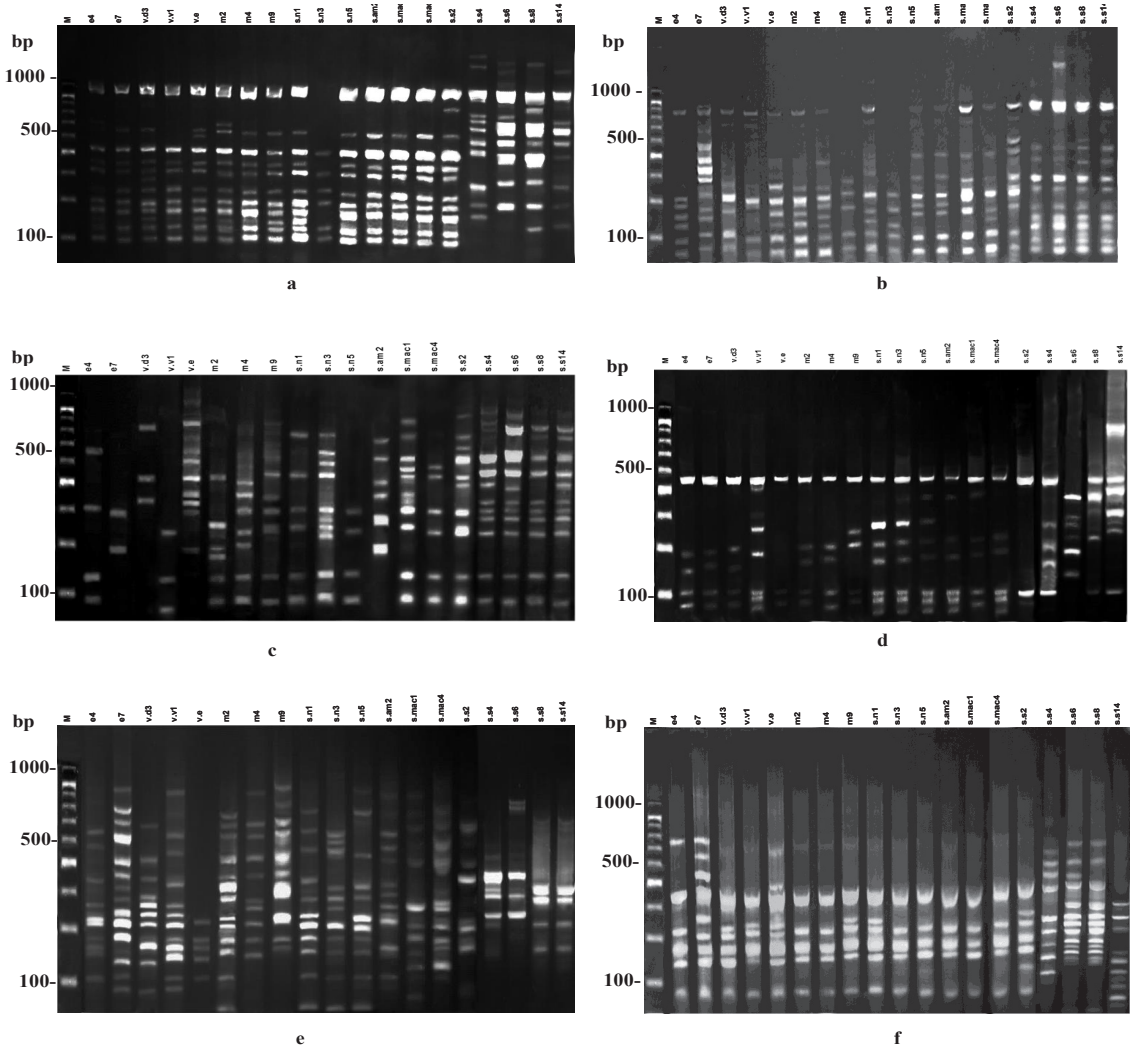

Fig. 1. Amplification products in *Vicia* species using six primers: a. ISSR-1, b. ISSR-3, c. ISSR-4, d.ISSR-6, e. ISSR-9, f. ISSR-10. M: 1kbp DNA ladder [See table 1 for abbreviations of the 19 accessions of *Vicia*]

TABLE 3. Comparative analysis of the six ISSR primer results of 19 accessions of different *Vicia* species. TAB: Total Amplified bands, Pb: Polymorphic bands, UB: Unique band

Primer Results	e4	e7	v.d3	v.v1	v.e	m2	m4	m9	s.n1	s.n3	s.n5	s.am2	s.mac1	s.mac4	s.s2	s.s4	s.s6	s.s8	s.s14
Primer 1: ∑ scorable bands= 29																			
TAB(no.)	6	10	12	9	11	12	10	11	11	6	10	11	11	11	12	11	10	10	10
Pb (no.)	6	10	12	9	11	12	10	11	11	6	10	11	11	11	12	11	10	10	10
Pb (%)	20.7	34.5	41.4	31.0	37.9	41.4	34.5	37.9	37.9	20.7	34.5	37.9	37.9	37.9	41.4	37.9	34.5	34.5	34.5
UB (no.)	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0
Primer 3: ∑ scorable bands= 25																			
TAB (no.)	7	11	6	4	7	7	9	5	7	5	8	8	8	7	9	10	11	11	10
Pb (no.)	6	10	5	3	6	6	8	4	6	4	7	7	7	6	8	9	10	10	9
Pb (%)	24	40	20	12	24	24	32	16	24	16	28	28	28	24	32	36	40	40	36
UB (no.)	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Primer 4: ∑ scorable bands= 28																			
TAB (no.)	4	2	3	3	9	6	7	9	6	10	4	6	11	6	10	12	11	10	11
Pb (no.)	4	2	3	3	9	6	7	9	6	10	4	6	11	6	10	12	11	10	11
Pb (%)	14.3	7.1	10.7	10.7	32.1	21.4	25.0	32.1	21.4	35.7	14.3	21.4	39.3	21.4	35.7	42.9	39.3	35.7	39.3
UB (no.)	0	0	0	1	0	1	1	0	0	3	0	0	0	0	1	0	1	0	0
Primer 6: ∑ scorable bands= 25																			
TAB (no.)	5	5	5	8	3	5	5	5	7	7	8	6	6	6	3	5	6	5	7
Pb (no.)	5	5	5	8	3	5	5	5	7	7	8	6	6	6	3	5	6	5	7
Pb (%)	20	20	20	32	12	20	20	20	28	28	32	24	24	24	12	20	24	20	28
UB (no.)	1	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0	2	0	1
Primer 9: ∑ scorable bands= 36																			
TAB (no.)	9	14	10	12	5	12	10	10	10	10	10	10	10	10	5	6	5	4	4
Pb (no.)	9	14	10	12	5	12	10	10	10	10	10	10	10	10	5	6	5	4	4
Pb (%)	25.0	38.9	27.8	33.3	13.9	33.3	27.8	27.8	27.8	27.8	27.8	27.8	27.8	27.8	13.9	16.7	13.9	11.1	11.1
UB (no.)	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0
Primer 10: ∑ scorable bands= 25																			
TAB (no.)	6	9	6	6	8	5	5	6	6	5	5	5	5	5	7	9	12	12	9
Pb (no.)	6	9	6	6	8	5	5	6	6	5	5	5	5	5	7	9	12	12	9
Pb (%)	24	36	24	24	32	20	20	24	24	20	20	20	20	20	28	36	48	48	36
UB (no.)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	3

(See table 1 for species abbreviations)

The efficiency of the chosen ISSR primers was slightly different and is summarized in Table 4. The six ISSR primers produced a total of 888 bands across the 19 examined genotypes (accessions) of all species, of which 168 were total scorable polymorphic bands including 1 monomorphic band for all primers, accounting for high polymorphism (99%). The PIC of ISSR primers ranged from 0.87 (ISSR-1) to 0.95 (ISSR-6), with an average of 0.92 irrespective of the number of total scorable bands or percent of polymorphism. The MI values ranged from 87 to 95 (for ISSR-1 and ISSR-6, respectively), with an average of 91.7 per primer. The resolving power (Rp) values ranged from 8.6 for primer ISSR-6 to 13.2 with ISSR-1 with an average of 10.6 per primer. The primers that showed higher polymorphism were observed to have a higher EMR. This feature varied from 131 to 194, with a mean value of 147.

Genetic diversity analysis and differentiation at species and subspecies level

Genetic diversity parameters of ISSR data using the POPgene software are presented in Table 5. To apply this analysis, *V. sativa* subsp. *amphicarpa* and *macrocarpa* were grouped together to increase the sample size. We found the highest observed number of alleles (na), and the most effective number of alleles (ne) were in *V. sativa* subsp. *sativa* (1.55, 1.37, respectively), whereas the lowest were in *V. ervilia* with an equivalent number of observed and effective number of alleles (na=ne= 1.26). All the other examined species and subspecies showed nearly the same observed number of alleles. The highest Nei's genetic diversity (h) and Shannon's information index (I) were for *V. sativa* (0.17 and 0.32, respectively) and the lowest for *V. ervilia* (0.13 and 0.18, respectively). However, subspecies *sativa* showed the highest value (0.21) among the four subspecies. The overall genetic diversity parameters of the seven studied *Vicia* species were estimated from all populations by the six ISSR primers .Our results indicated that were about 2 na and there were 1.4 ne, giving a Nei genetic diversity value of 24% and a 39% Shannon' s information index. The analysis of gene diversity in subdivided populations of these studied species, showed 23% total gene diversity (Ht) and 11% gene diversity within populations (species) (Hs) with a coefficient of gene differentiation (Gst) of 54%.

The value of Nei' s genetic identity varied from 0.75 between *V. ervilia* and *V. sativa* subsp. *amphicarpa* to 0.90 between *V. villosa* and *V. sativa* subsp. *nigra* (Table 6). The average genetic identity in all species was 0.61, while that of *V. sativa* complex was 0.20. Based on Nei's genetic distance extracted from the previous matrix of table 6, a dendrogram was constructed (Fig. 2). This revealed that *V. sativa* subsp. *amphicarpa* differed in terms of ISSR characteristics from the other species acting like anout group. Notably, *V. sativa* subsp. *nigra*, *V. monantha* and *V. villosa* formed one cluster.

ITS sequence diversity and GC content

The ITS1 and ITS4 primer amplification of the nrDNA region (ITS1-5.8S-ITS2) of the ten *Vicia* accessions yielded a single band around 700 bp. In this investigation, partial 18S, complete ITS1-5.8S-ITS2, and partial 28S sequences were recovered. The whole ITS region ranged in length from 631 bp in *V. villosa* subsp. *villosa* to 753 bp in *V. sativa* subsp. *sativa* (Table 7). The GC content was about 50%. In general, it was between 47.39 percent and 53.59 percent in *V. villosa* subsp. *villosa* and *V. sativa subsp*. *nigra*, respectively.

The consensus sequences of all accessions were aligned using Clustal X2. This analysis revealed that out of the 799 characteristics evaluated, 89 were preserved across all accessions. Those of the *V. sativa* complex, on the other hand, shared 391 out of 780 characteristics, while the 2 *V. sativa* accessions ss2 and ss14 shared 713 out of a total of 780 characteristics. Of 741 characteristics, the 3 *V. villosa* subspecies shared 122 characteristics. Due to gaps, certain accessions were 9–80 nucleotides shorter than the average nucleotide length (711bp) of the other *Vicia* species (Table 7). When the ITS base sequences of this current study' s accessions were compared to nucleotide sequences derived from NCBI data we found that *V. ervilia, V. monantha, V. sativa* subsp. *amphicarpa*, *V. sativa* subsp. *sativa*, and *V. villosa* subsp. *eriocarpa* had identities ranging from 91 to 99 %. The sequencing data for all the examined *Vicia* accessions has been deposited in GeneBank/NCBI under accession MW 540811-540820.

TABLE 4. The efficacy of ISSR primers used with the studied *Vicia* accessions

Primer	Sequence	Primer motif	CG%	Band size (kb) range	SC.B	Amplified bands			Pb%	UB%	PIC	MI	RP	EMR
						TAB	MB	UB						
ISSR-1	5'CA CA CA CA CA CA GG3'	$(CA)_6GG$	57	83-1217	29	194	0	4	100.0	2.1	0.87	87	13.2	194
ISSR-3	5'GT GT GT GT GT GT GG3'	$(GT)_6GG$	57	62-1223	25	150	1	2	99.3	1.3	0.92	91.4	12	148
ISSR-4	5'GA GA GA GA GA GA CC3'	$(GA)_6CC$	57	66-829	28	140	0	8	100.0	5.7	0.92	92	10	140
ISSR-6	5'CAC CAC CAC GC3'	$(CAC)_3GC$	73	64-822	25	107	0	8	100.0	7.5	0.95	95	8.6	107
ISSR-9	5'CA CA CA CA CA CA AG3'	$(CA)_6AG$	50	70-845	36	166	0	2	100.0	1.2	0.93	93	9.2	166
ISSR-10	5'CT CT CT CT CT CT CT CT GC3'	$(CT)_8GC$	56	65-651	25	131	0	5	100.0	3.8	0.92	92	10.5	131
Total					168	888	1	29	599.3	21.6	5.51	550.4	63.5	886
Mean					28	148	0.2	4.8	99.9	3.6	0.92	91.7	10.6	147.7

SC.B: Scorable bands, TAB: Total amplified bands for all accessions, PB: Polymorphic bands, MB: Monomorphic (common) bands, UB: Unique bands, P%: Percent of polymorphism, UB%: Percent of unique bands, PIC : Polymorphic information content, MI: Marker index, RP: Resolution power, EMR : Effective multiplex ratio.

TABLE 5. Detailed genetic diversity parameters in *Vicia* species based on ISSR markers for the 6 studied populations.

Population name	Number of accessions	P	P %	na	ne	h	I
V. ervilia L.	2	43	25.38	1.2619	1.2619	0.1310	0.1815
V. monantha Retz	3	45	26.51	1.3214	1.2571	0.1429	0.2046
V. villosa Roth	3	41	24.38	1.3155	1.2524	0.1402	0.2008
V. sativa L. subsp. *amphicarpa* **and** *macrcarpa*	3	46	27.29	1.3333	1.2667	0.1481	0.2122
V. sativa L. subsp. *nigra*	3	44	25.99	1.3214	1.2571	0.1429	0.2046
V. sativa L. subsp. *sativa*	5	50	51.46	1.5476	1.3681	0.2143	0.3162
Total and mean for *V. staiva*	11	141	34.91	1.40	1.30	0.17	0.24

P: Number of polymorphic loci, P %: Percent of polymorphic loci, na: Observed number of alleles, ne: Effective number of alleles, h: Nei's genetic diversity, I: Shannon's information index.

TABLE 6. Nei's original measures of genetic identity and genetic distance (Nei, 1972) among the different studied species using ISSR data analysis

Pop ID	e	v	m	s.n	s.am	s.mac	s.s
e	-	0.9138	0.8919	0.878	0.7485	0.8198	0.833
v	0.0901	-	0.9337	0.9049	0.7679	0.8152	0.825
m	0.1144	0.0686	-	0.9057	0.7665	0.8263	0.8203
s.n	0.1302	0.0999	0.0991	-	0.8001	0.8765	0.8614
s.am	0.2897	0.264	0.2659	0.223	-	0.8041	0.7618
s.mac	0.1988	0.2044	0.1909	0.1318	0.218	-	0.8153
s.s	0.1827	0.1924	0.1981	0.1492	0.2721	0.2042	-

Nei's genetic identity (above diagonal) and genetic distance (below diagonal)

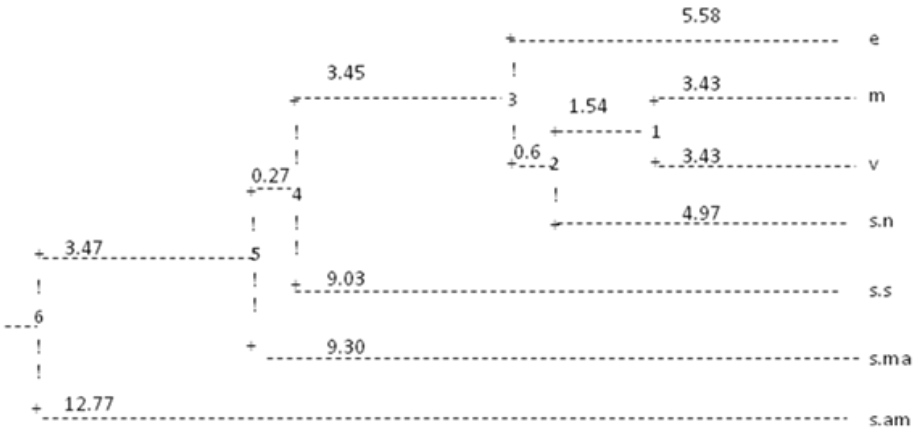

Fig. 2. Dendrogram based on Nei's (1972) genetic distance of different studied *Vicia* species using ISSR data: UPGMA modified from neighbor joining procedure of phylip ver.3.5.

TABLE 7. Aligned of ITS regions (dervived from primer ITS4) of the 10 studied accession of the present study using Clustal X2 and BioEdit softwarers

Species	Accession code	Length of ITS region (bp)		Gaps (absent nucleotides)	G+C content (%)
		without gaps	with gaps		
V. ervilia	e4	741	792	51	51.01
V. monantha	m9	721	767	46	50.49
V. sativa subsp. *nigra*	s.n5	752	793	41	53.59
V. sativa subsp. *amphicarpa*	s.am2	678	721	43	50.44
V. sativa subsp. *macrocarpa*	s.mac4	676	721	45	49.56
V. sativa subsp. *sativa*	s.s2	760	799	39	50.79
V. sativa subsp. *sativa*	s.s14	753	796	43	51.79
V. villosa subsp. *dasycarpa*	v.d3	702	739	37	50.28
V. villosa subsp. *villosa*	v.v1	631	696	65	47.39
V. villosa subsp. *eriocarpa*	v.e	700	779	79	50.86

ITS phylogeny

Phylogenetic relationships among the studied accessions were constructed with the MEGA version 4 program using different distances models and algorithms. Branch reliability was assessed by bootstrapping the data with 1000 replicates. Simulation analysis was carried out for cluster analysis using the five outgroup ITS

sequences of one Lens species *(Lens culinaris)* and four *Lathyrus* species (*L. laxiflorus*, *L. pratensis*, *L. clymenum* and *L. Spathulatus)* from GeneBank/ NCBI. We found that the substitution of varying distances and algorithms with or without the combinations of the additional outgroups did not affect tree topology. As shown in Fig. 3, all accessions were clustered into two groups (clades). Group I included all *sativa* complexes as sister taxa with a high bootstrap value (98%) except subsp. *nigra*. Group II included *V. sativa* subsp. *nigra, V.monantha,* and *V. ervilia* with moderately high bootstrap support (90%). Both groups were paraphyletic to *V. villosa* subsp. *dasycarpa* as an outgroup, and all accessions were paraphyletic to the other two *V. villosa* subspecies. Using the five outgroup taxa, *V. villosa* subsp. *villosa* acted as a monophyletic cluster to the other eight accessions including *V. villosa* subsp. *dasycarpa* with a 100% bootstrap value. However, *V.villosa* subsp. *eriocarpa* acted as outgroup rooted species with short branch length.

The pairwise comparison of nucleotide substitution indicated more transversions than transitions among the nucleotides of the ten *Vicia* accessions using the parsimony approach and the probability parameters of the PAUP software (Table 8). In total, 9 of the 45 pairwise comparisons yielded the highest transition/transversion (Ti/Tv) ratio of 1.1, while the 4 closely related *V. sativa* subspecies yielded a mean ratio of 0.89, and those of *V. villosa* yielded a mean ratio of 0.79.

Appling the parsimony, heuristic search to the aligned sequences of the 10 accessions without outgroups resulted in 799 characters. There were 642 variable characters of which 410 were parsimony uninformative (~51%) and 232 characters (29%) contained potential phylogenetic information. The parsimonious analysis of the present ITS sequence data resulted in 100 equally parsimonious trees with and without outgroups. The strict consensus trees using the heuristic, branch- and bound-, and likelihood analysis separately gave identical tree topology and differed only by their bootstrap values (Fig. 4). We observed the *V. sativa* complexes except four subsp. *nigra* were clustered together as did the *V. villosa* subspecies, while *V. ervilia* and *V. monantha* were paraphyletic to them Parsimony analysis with Accelerated Transformation character state optimization (ACCTRAN) yielded a tree length of 1049 steps. After excluding the uninformative characteristics, the consistency index (CI) was 0.7122, and the homoplasy index (HI) was 0.2878. The same topology for clustering was found in the parsimony analysis with the five outgroup species but with a tree length of 1231 steps (CI= 0.8076, HI= 0.1924) (Fig. 4b). These PAUP trees were compatible and slightly less resolved than the strict consensus tree from the MEGA analysis for the placement of *V. sativa* subspecies *nigra.*

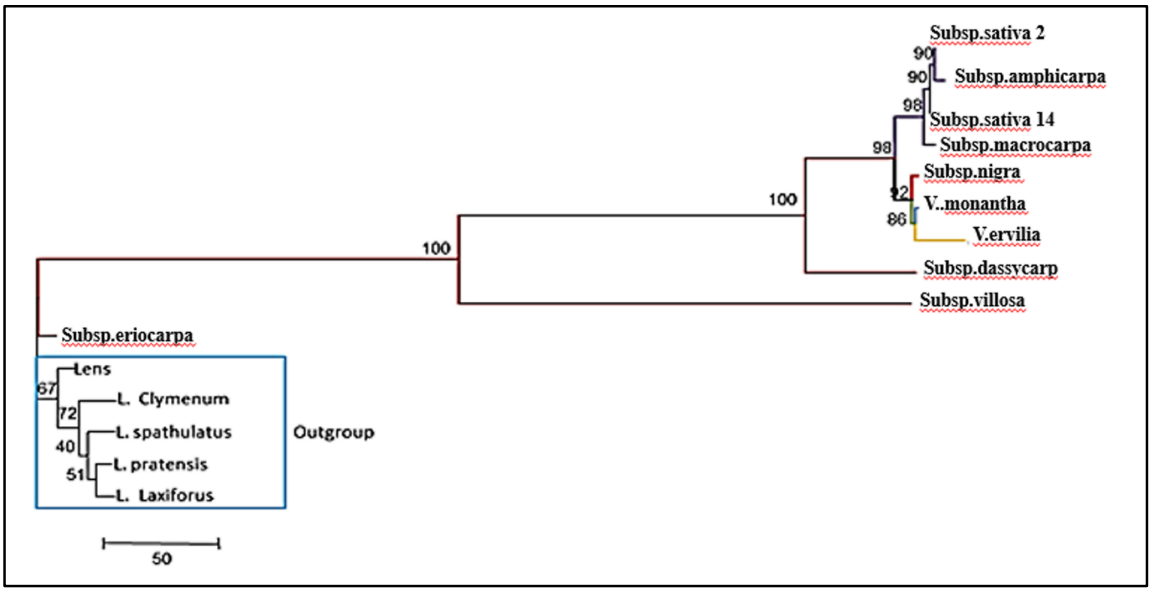

Fig. 3. Phylogenetic trees inferred from ITS sequences of 10 *Vicia* accessions with outgroups generated by the Maximum Composite Likelihood model using MEGA software [Numbers above branches are bootstrap values]

TABLE 8. Pairwise comparison of nucleotide substitutions (Transition Ti/ transversion Tv) of the 10 ITS sequences of the studied *Vicia* species

	e4	m9	s.n5	s.am2	s.mac4	s.s2	s.s14	v.d3	v.v1	v.e
e4	-	29/50	29/52	40/38	33/35	45/56	51/53	57/109	141/191	180/218
m9	0.58	-	31/29	32/30	29/27	34/43	33/47	59/91	141/188	177/211
s.n5	0.56	**1.07**	-	28/25	21/28	34/63	88/59	61/91	144/187	175/223
s.am2	**1.05**	**1.07**	**1.12**	-	17/16	11/15	11/14	65/78	139/195	166/209
s.mac4	0.94	**1.07**	0.75	**1.06**	-	13/12	14/13	61/82	140/189	163/207
s.s2	0.80	0.79	0.54	0.73	**1.08**	-	23/21	66/92	136/194	182/230
s.s14	0.96	0.70	0.64	0.79	**1.08**	**1.10**	-	68/86	136/192	177/232
v.d3	0.52	0.65	0.67	0.83	0.74	0.72	0.79	-	147/198	164/219
v.v1	0.74	0.75	0.77	0.71	0.74	0.70	0.71	0.74	-	181/205
v.e	0.83	0.84	0.78	0.79	0.79	0.79	0.76	0.75	0.88	-

Direct counts of transitions/ transversions (above diagonal) and their ratio (below the diagonal). (See table 1 for abbreviations).

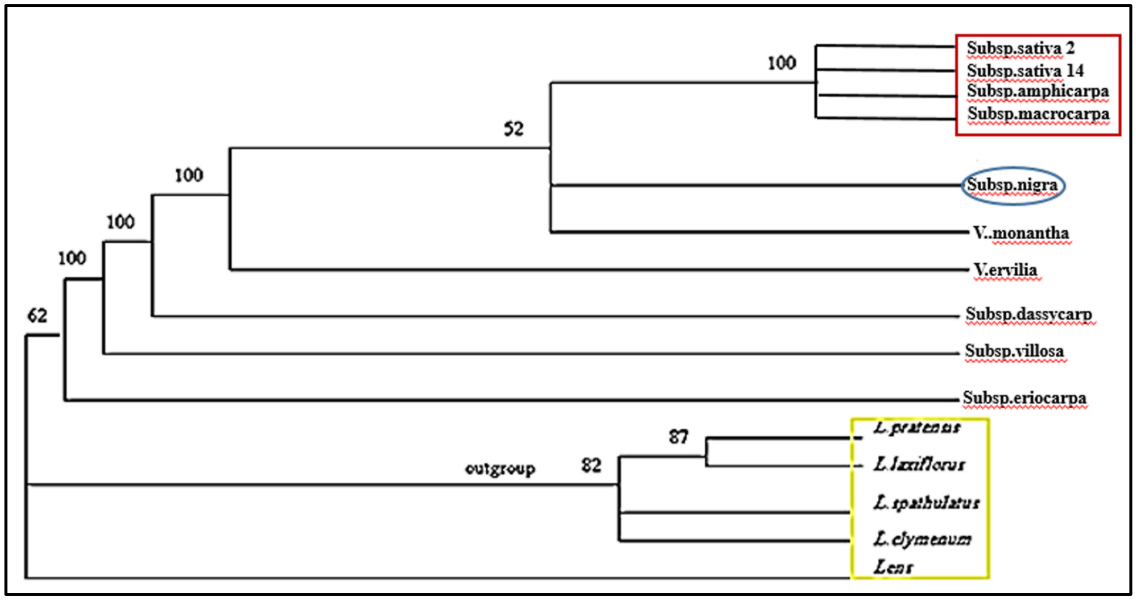

Fig. 4. Parsimony tree inferred from ITS sequences of the 10 *Vicia* accessions generated with outgroup using PAUP software. Bootstrap values more than 50% are shown [Tree length= 1231 steps, (CI= 0.8076 and HI= 0.1924, excluding uninformative characters, goodness of fit statistics of value= 1956 and f ratio= 0.1296)]

Discussion

Molecular markers are useful because they can identify primarily selectively neutral alterations at the DNA level. Their presence is unaffected by the environment or the stage of plant growth, thus making them ideal for genetic association studies between species and genotypes (Reddy et al., 2002; Chakravarthi & Naravaneni, 2006). Omondi et al. (2016) stated that ISSR analysis was sensitive and gave reproducible polymorphism information, beside it being rapid and cost effective. ISSR, a molecular method, was employed in this investigation to identify heterogeneity among and within 19 *Vicia* accessions.

In this study we were able to develop 888 distinct, reproducible ISSR bands demonstrating the efficiency of the 6 ISSR primers in generating the high number of characteristics in the studied 19 *Vicia* accessions. Primers used in this study were high in GC content (50% –73%), ensuring more stable binding of primer/template compared to A-T bonds (Mitsuhashi, 1996). Herein, primers with poly "CA" motifs generated a larger number of amplified loci than those with poly "GA" motifs. This finding contradicted that of De la Torre

et al. (2012). Additionally, dinucleotide motif primers gave a higher number of total amplified loci than the trinucleotide motif indicating that the dinucleotide motifs are more amenable to ISSR analysis as suggested by Blair et al. (1999). However, the trinucleotide motif primer (ISSR-6) is noted to be shorter than the others, which could possibly be an additional cause of being inefficient in priming a high number of loci. Here, primers ISSR-1 and ISSR-9 had the same motif but differed in one nucleotide on the anchor bases. The former' s anchor bases were GG, which gave the highest total amplified bands for all accessions (194 loci) and 29 scorable loci; latter' s anchor bases were AG, which gave 166 total amplified loci for all accessions and 36 scorable loci. The variability in binding sites throughout the genomes of the examined genotypes could explain the variation in the number of loci created by these random primers as suggested by Rizza et al. (2007).

The statistical measurements of ISSR primer efficacy utilized in this study indicated that the ISSR primers used are deemed very informative. With a mean value of 0.92, the PIC was high. Many genetic diversity studies have used this parameter extensively (Tatikonda et al., 2009; Talebi et al., 2010; Thudi et al., 2010). With a mean of 91.73, a high PIC index for a marker occurred with a high MI, indicating a high level of polymorphism. This high level of polymorphism was mirrored in the EMR, which had a mean of 147. Powell et al. (1996) and Nagaraju et al. (2001) found similar results. In some plant species, the EMR and MI have been employed to assess the discriminatory strength of molecular marker systems (Kumar et al., 2009; Tatikonda et al., 2009; Thudi et al., 2010; Etminan et al., 2018). According to Prevost & Wilkinson (1999), RP index provides a reasonable approximation of how many genotypes a primer can identify. Here, two of the ISSR primers (ISSR-1 and ISSR-3) had high RP values (13.2 and 12, respectively) and hence appeared to be the most successful primers for genotype separation. Because the discriminating power of a primer increases as the number of bands generated by that primer increases, PIC is inversely correlated with both MI and RP. In conclusion, the efficacy of the six primers employed in evaluating the various *Vicia* genotypes investigated is high.

The cluster analysis supported the ability of ISSR to represent the genetic structure of the examined accessions. A standard accession reference array, based on genetic similarity estimations, may be required, which could comprise unique accessions such as s.s2 of *V. sativa* subspecies *sativa*. The premise of the Shannon index considers both abundance of diversity and evenness of species present in the community. It revealed that *V. sativa* subspecies *sativa* showed the highest genetic diversity parameters, while the lowest values were found for *V. ervilia*. Becuse the interspecies diversity (Gst) was 0.54, it can be assumed that a high percentage of genetic diversity is distributed among the examined species. However, the intrapopulation genomic diversity of the studied species was still higher than interpopulation diversity in some cases such as *V. sativa* subsp. *sativa*. Similar conclusions for Fabaceae were reported by Bulinska-Radomska (2000) and Isobe et al. (2009).

One of the most used loci in molecular phylogenetic analysis of angiosperms is the internal transcribed spacer (ITS1-5.8-ITS2) of the nrDNA region (Markos & Baldwin, 2001). In our study, ten of the four *Vicia* species and subspecies were used to examine the nucleotide sequence of the ITS region of nrDNA. Results showed that this region was 700 bp in *V. villosa* subsp. *eriocarpa* and up to 753 in *V. ervilia*, *V. monantha*, *V. sativa* subsp. *nigra, V. sativa* subsp. *sativa,* and *V. villosa* subsp. *dasycarpa.* However, this region was 678, 676 and 631 bp in *V. sativa* subsp. *amphicarpa*, subsp. *macrocarpa*, and *V. villosa* subsp. *villosa* respectively. These findings contrasted with those of Baldwin et al. (1995), who claimed that in flowering plants, the entire ITS region appeared to be below 700 bp. They also noted that the ITS region of *Vicia* was only about 607 bp. However, ITS length variation was reported earlier by Jorgensen & Cluster (1988) to be as much as 200 bp in nine Legume genera extracted from seven tribes. In contrast, sequenced ITS regions of grass species were found by Hsiao et al. (1993) to be relatively conserved and have no length variation. Point mutations as deletion and insertion events (indels) rather than length mutations may be responsible for the differences in ITS region lengths among those closely related species causing gaps in the sequence alignment (Scoles et al., 1988; Baldwin, 1993; Hsiao et al., 1993). The insertion of gaps during the process of automated alignment was necessary to preserve the potential nucleotide homology during sequence alignment. Later in 2005, it was suggested that tandem

repeats causing non-homologous recombination and/or unequal crossing-over events can also lead to ITS length variation (Won & Renner). Our analysis confirmed that there is a considerable polymorphism in the length of the ITS region as it was not highly conserved in these studied species and sequence diversity was recorded among them.

The average GC content of ITS regions of the studied species and subspecies was around 50% in several groups of angiosperms. This GC content was comparable to the values recorded by Yokota et al. (1989) in *V. faba,* as quoted by Baldwin et al. (1995). However, the highest value was for *V. sativa* subsp. *nigra*, whereas the lowest values (<50%) were for *V. sativa* subsp. *macrocarpa* and *V. villosa* subsp. *villosa.* The shift in GC content among related accessions was suggested by Baldwin et al. (1995). They suggested that it could indicate a bias in substitution probabilities, which could make homoplasy detection more difficult in these sequences than in those with more balanced substitution expectations.

The variation among ITS sequences could be attributed to insertion/deletion (indel) mutations as well as transition and transversion point mutations. The alignment of ITS sequences requires the insertion of gaps where indel mutations occurred to preserve nucleotide positional homologies (Baldwin et al., 1995). Because of the flanking conserved sites, placement of gaps in most indel regions proved unambiguous. In general, sequence alignment is aided by the interspersion of conserved and variable characters in the ITS region, and indels can be viewed as a scope rather than a barrier in the alignment process. However, transition and transversion point mutations can shed light on the evolutionary process of the examined accessions. We found that the ratios of transitions to transversions (Ti/Tv) were higher among *V. sativa* subspecies than among the *V. villosa* subspecies. The mean ratio of Ti/Tv in the 10 ITS sequences was 0.831 (range from 0.5397 to 1.1), which is significantly lower than Holmquist's (1983) 2.0 estimate for recently diverged sequences and higher than the value of 0.4 for high substitution saturated sequences. This result might suggest that species (accessions) with low Ti/Tv ratios have a longer evolutionary history than those with high Ti/Tv ratios. Looking at the entire picture of the Ti/Tv ratio, three key tendencies emerged. **First**, *V. ervilia* and each of *V. monantha, V. sativa subsp. nigra*, and *V. villosa* subsp. *dasycarpa*, as well as *V. sativa* subsp. *nigra* and subsp. *sativa* (s.s2), showed a trend with extremely low values (0.5 %). This result suggests that they have a longer evolutionary history, or in other words, primitively split in the phylogenetic tree**. The second trend** showed a tendency toward higher values of transitions (~1.1%) and was characteristic of the pairs *V. sativa* subsp. *sativa* and subsp. *macrocarpa, V. sativa* subsp. *macrocarpa* and subsp. *amphicarpa, V. sativa* subsp. *nigra* and subsp. *amphicarpa, V. ervilia* and *V. sativa subsp. amphicarpa, V. monantha* and each of *V. sativa* subsp. *nigra,* subsp. *amphicarpa* and subsp. *macrocarpa.* This trend signifies the recent evolutionary split of this *sativa* complex species. **The third trend** comprised the remaining pairwise comparisons with values ranging from 0.5 to 1.0. Here, transversions were not to occur more than transitions, explaining lower homoplasy among these pairs according to Graur & Li (2000). This tendency existed according to the presence of eight possible methods for transversion compared to four possibilities for transitions. As a result, transversions were thought to be a more dependable sort of mutation when constructing phylogenies. Consequently, some studies either gave more weight to transversions in phylogenetic analyses or the analysis was based on transversions alone. This bias results in what is called transversion parsimony (Lake, 1987 and Quicke, 1993 as quoted by Saha et al., 2013).

The ITS sequences of the ten *Vicia* accessions were analyzed for a phylogenetic signal using the random tree option in PAUP 4 to determine the skewness (g_1 value) of a random tree_length distribution. The 1000 random parsimony 'trees' tree_length distribution was found to be significantly skewed (g1= -1.38). This value indicates that there is a high likelihood of a phylogenetic signal in the data set. Phylogenetic trees with congruent and consistent topologies were generated using various statistical analyses such as MEGA and PAUP ensuring that the current results are consistent and accurate, as stated by Ballard et al. (1992).

The phylogenetic tree of both analysis methods indicated two lineages within the *V. sativa* species complex. All *V. sativa* complex accessions belonged to one lineage, except for *V. sativa* subsp. *nigra* (accession from Egypt). It formed the other lineage and was placed in the same lineage by Shiran & Raina (2001). In this

current study, the bootstrap phylogeny test clearly revealed that the three subspecies of *V. sativa* (*sativa*, *amphicarpa*, and *macrocarpa*) belonged to one evolutionary branch with a high bootstrap value (98% –100%), indicating that they were closely related. In the MEGA analysis, subspecies *nigra* persisted as a single monophyletic branch closer to *V. ervilia* and *V. monantha*, but they branched separately in the PAUP analysis. The effect of outgroup on species branching pattern has been thoroughly demonstrated in parsimony investigations (Baum & Estabrook, 1996; Roy, 2009). However, neither of these trees, which were based on the current study' s outgroup species, was beneficial in determining the ancestry of *V. sativa* subsp. *nigra*.

Some insights can be gleaned from our results; subsp. *nigra* is genetically distinct from the other subspecies of *V. sativa*. Its phylogenetic position can be a challenge in identifying whether *sativa* is mono- or paraphyletic. Using the PAUP program, all *V. villosa* subspecies stayed clustered in the same way as the *sativa* complex (except for subsp. *nigra*). In all trees, the g_1 statistic value is negative, suggesting that the tree length distribution is skewed to the left. Thus, the corresponding phylogenetically informative characters underwent little or almost no parallel or convergent evolution on different branches of the molecular tree. This result means moderate homoplasy, and characters were not strongly shared from a common ancestor. Sanderson & Hufford (1996) confirmed that CI measures aspects of the goodness of fit of a data set to a hierarchical tree structure.

Conclusion

In conclusion, the important outcome of this study is that the diversity of the ITS region among the species of *Vicia* is extensive and provides sufficient resolution to solve some questions of the phylogenetic tree patterns obtained through ISSR analysis. The combined types of analysis (ISSR and ITS) resulted in several trees that are congruent to some extent. The similar topology and high bootstrap values of ITS trees have lead us to the final conclusion that s.s2 was nested with their corresponding subsp. *sativa*. However, accession s.n5 of subspecies *nigra* from Egypt must have unique characteristics in its gene pool compared to most other wild studied accessions of *Vicia.*

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Author's contribution: Ghada E. El-Badan; performed the measurements, processed the experimental data using different universal computer programs to validate the data and writing the original draft, Laila M. El-Sadek; suggested the study and supervised it, reviewed the paper and conceived the presented idea, Amal W. Amin; supervised the study, Data curation and reviewed the paper, Fatma M. Ashour; supervised and reviewed the paper, All authors discussed the results and contributed to the final manuscript.

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توصيف التنوع الجيني لبعض أنواع جنس الفشيا باستخدام التقنية الجزيئية ISSR , ITS

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أدت الأهمية الاقتصادية العالية لجنس الفشيا إلى مجموعة كبيرة من الدراسات حول التوصيف الجزيئي والتحقيق في العلاقات التطورية بين الأنواع التي تنتمي إلى هذا الجنس. تعتبر منطقة البحر الأبيض المتوسط هي المركز الرئيسي للتوزيع والتنوع حيث ارتبط تعدد الأشكال بالأصل الجغرافي للمحتوي الجيني لهذه الأنواع (إيكاردا، 2013). يمكن تقييم مدى التنوع الجيني في للمحتوي الجيني من خلال التوصيف المورفولوجي والواسمات الجينية. تتمتع التقنيات الجزيئية بتقييم أكثر دقة للمكونات الجينية والبيئية للتنوع، مما يوفر دقة أكبر في مقاييس التنوع الجيني.

الدراسة الحالية عبارة عن تحليل جزيئي للأصول الوراثية لفيشيا من خلال تقنيات ISSR و ITS. كانت الأهداف هي تحديد التنوع الجيني وعلاقة النشوء والتطور بين 19 نوع أو سلالة برية، جمعتها هيئة الإيكاردا من أماكن جغرافية مختلفة ، تمثل تسعة أنواع وتحت أنواع فرعية من:
V. ervilia; V. monantha; V. villosa subsp; *villosa*, *dasycarpa, eriocarpa*; *V. sativa* subsp; *nigra*, *amphicarp*, *macrocacrpa*, *sativa*

تمت دراسة أنماط التباين في هذه السلالات من خلال تحليل للبيانات للوصول لمعلومات وراثية ذات صلة بالاصول الوراثية وتطور نشأة السلالات وهذا أدى لاستكمال بيانات جواز السفر للمجموعات تحت الدراسة تفيد المركز العالمي للبحوث الزراعية في الاراضي الجافة.