Genetic diversity and relationship among (Vicia faba L.) Using SSCP –PCR- Sequencing Techniques

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Abstract

Since their potential and genetic background were not thoroughly investigated, Algerian accessions constitute a crucial source of features of interest for crop enhancement, particularly for combating climate change. To evaluate the relationships and genetic diversity among 3 collected vicia faba isolated from various parts of Iraq, target region amplification polymorphism markers have been employed. For resolving genetic linkages across populations and to determine the genetic diversity of rare and endemic species, SSCP-PCR is helpful. Single-strand conformation polymorphism and polymerase chain reaction (PCR) have been utilized in order to genotype ITS4 gene. As a result, DNA sequencing was used for confirming such DNA polymorphisms. Depending on the SSCP-PCR method's results, there have been three distinct haplotype patterns present, with bands having bands of 4, 3, and 2 respectively. Yet, establishing the pattern regarding all resolved SSCP bands only through gel visualization might be challenging. After that, DNA sequencing should be used to confirm these DNA polymorphisms. According to the sequence's results, multiple SNPs were used to identify ITS region's haplotypes. All 3 sequenced isolates were deposited in GenBank at DNA Data Bank Japan (DDBJ) database with accession numbers (LC733679, LC733680, LC733681).

Conclusion: our research showed that the SSCP-PCR method is more useful for the assessment of the relation and genetic diversity among Vicia faba L populations.

Keywords: *PCR, SSCP, polymorphism, vicia faba and sequencing.*

Introduction

Winter crops like faba bean (Vicia faba L.) are crucial. This legume, which has its origins in West Asia and the Mediterranean (Willcox and Tann, 2006), is becoming more and more well-known because of its large amount of biomass, high yield potential, high protein content of seeds, and the capability for fixing nitrogen in

soil (Angra and O'Sullivan, 2016). Faba bean is ranked amongst cool season grain legumes, coming in behind peas, cowpeas, lentils and chickpeas (faostat,2021).

Faba bean grain with large seeds is normally consumed as food, whereas medium-sized grains are commonly consumed as both feed and food and small-sized grain is majorly consumed as feed (Redden etal., 2007). Faba bean could be grown in turning with the cereal crops in order to enhance the physical condition of the soil, disrupt disease cycles, and manage weeds (Murray etal., 1998). Crop development projects must be established, managed, and ensured to be successful over the long term. This requires an understanding of a crop's relationships and genetic diversity between conserved germplasm collections (Ma etal., 2009; Gwak, 2008). Several genetic marker systems, such as isozyme (Mancinni etal., 1989), PCR, and random amplified polymorphic DNA (RAPD) (Link etal., 1995), as well as polymorphisms in restriction fragment length (inter-simple sequence repeat (Bebeli & Terzopoulos, 2008) and amplified fragment length polymorphism, were utilized in order to define the genetic diversity in the accessions of the faba beans (Zong etal., 2009). These techniques were helpful in explaining the relationships and genetic diversity between accessions in ex situ collections of faba bean germplasm. Genetic diversity of 79 elite North African, Asian and European types was documented by Zeid et al. in 2003. They used 8 AFLP primer combinations to amplify 477 polymorphic fragments, which can support information on history regarding faba bean dispersal and cultivation in researched areas and confirm some known pedigree relations. Zeid and colleagues (2003) discovered that Asian lines have been distinct as a group with the use of the principal coordinate analysis as well as unweighted pair group approach with arithmetic mean (UPGMA)-based clustering. Bebeli and Terzopoulos (2008) discovered a significant level of intrapopulation diversity up to 0.676 in their faba bean collection. Lately, Zong etal. (2009) reported on genetic diversity of AFLP-assessed winter faba bean germplasm from China and other parts of the world. It was discovered that the 39 accessions of varied geographic origins have been divided from 204 Chinese faba bean accessions.

The highly diversified rDNA (ribosomal DNA) ITS region could be utilized for analyzing the latest diversification of taxonomic areas or even within populations. When put to comparison with ancient diversification, rDNA has significantly preserved areas in the case of animals (Hyvönen and Pocza, 2010). On the other hand, the markers' nature utilized in earlier study has limited its scope (Rieseberg etal,1992). According to its definition, single-stranded polymorphic conformation analysis is a different approach for locating and genotyping genetic variations in different species. The key benefit of SSCP technique is that it is substantially less expensive compared to other genetic analyses and allows for the simultaneous sampling regarding the genetic composition of different species. The method presented here is applicable to both research and clinical laboratories and could be applied with very basic tools in very small laboratory setups (Chalmers et al 2005). This research's objective was to show how well the SSCP-PCr method worked for analyzing Vicia faba L'phylogenetic relationships separated from various regions of our nation.

Methodology

Sampling

Samples were taken between September 2021 and December 2021 in various parts of Iraq.

Genotypic identification

DNA Extraction

Utilizing "wizbio" extraction and clearing kit (south Korea), DNA of the plant Vicia faba L was extracted and purified.

Primers

IDTDNA (USA) was used for ITS region supplied from Bioneer. Specific primers for the SSCP-PCR method have been tested on the DNA of the Vicia faba L plant (table1).

Table1: Primer Sequences that have been utilized in the present research

Primers	Sequence 5----------3
ITS3	5- GCATCGATGAAGAACG CAGC-3
ITS 4	5- TCCTCCGCTTATTGATATGC-3

PCR amplification

25ul reaction volumes of 1ul forward and reverse primer, 12.5ul green Master, and 3ul were the final product. The genomic deoxyribonucleic acid and reaction rate were both rounded to 25 ul. Five mins of amplification were spent in an Eppendorf thermocycler. 35 cycles of 1min each at 94oC, 55oC, and 72oC with final extension of 10mins at a temperature of 72 Celsius were performed. One can reach a complete quantity at 35 cycles per minute. In 2% agarose gels, an amplification product has been electrophored, and ethidium bromide (EtBr) was used in order to visualize it. Standard molecular markers have also been used in each one of the electrophoresis runs. UV trans-illuminated gels were captured with photos.

SSCP- Sequencing

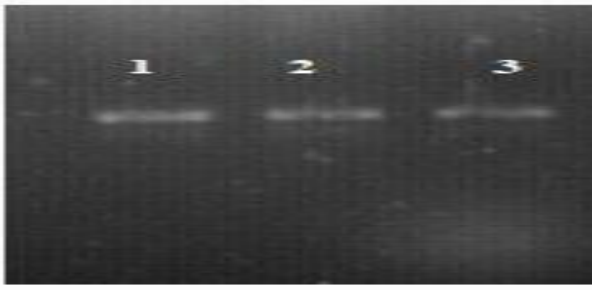
It was suggested that the SSCP goods must be run on minigels to save money and time. It could be challenging to determine the pattern regarding all resolved SSCP bands with the use of only gel visualisation. Sequencing of DNA is after that required to confirm DNA polymorphisms.

Results

Genotypes Of Vicia faba L Using Universal Primer

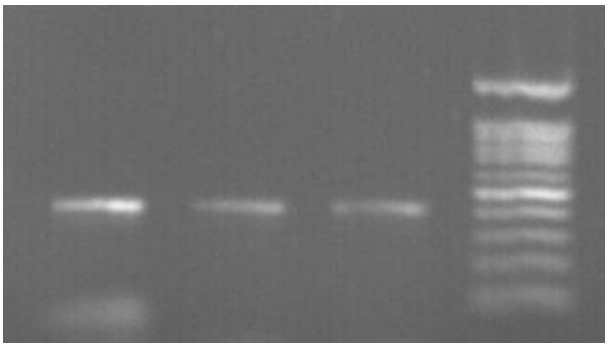
Genomic DNA has been amplified for general genotyping using certain primers and the thermocycling equipment under the ideal conditions outlined above. The outcomes showed that there was only single band present (400 bp).

Fig. (1): Electrophoresis pattern of the genomic DNA that had been extracted from vicia faba leaves samples.



Conditions of the Electrophoresis, 1% agarose, 75V, 20mA for 1hr (10µl in every one of the wells), stained with ethidium bromide

Fig. (2): Agarose gel electrophoresis of ITS4 amplified product (one sample from north of Iraq, sample2 from south and the last one from middle of Iraq).

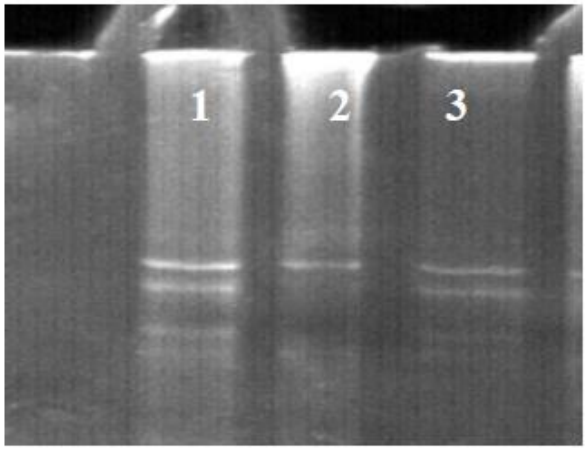


Electrophoresis conditions, 1% agarose, 75V, 20mA for 1hr (10µl in every one of the wells), which have been stained with the EtBr

PCR –SSCP

A PCR-SSCP method was used to genotype the ITS4 region after amplifying the target region. The PCR-SSCP gel electrophoresis (PCR-SSCP gel photo) results, given in figure (2), indicated that there were 3 unique haplotype models that have been identified by number of the bands. The single stranded DNA (ssDNA) bands at the gel's top and double stranded DNA (dsDNA) bands at gel's bottom were recognized. The genetic pattern regarding each amplified sample is ascertained using ssDNA variations in SSCP gels.

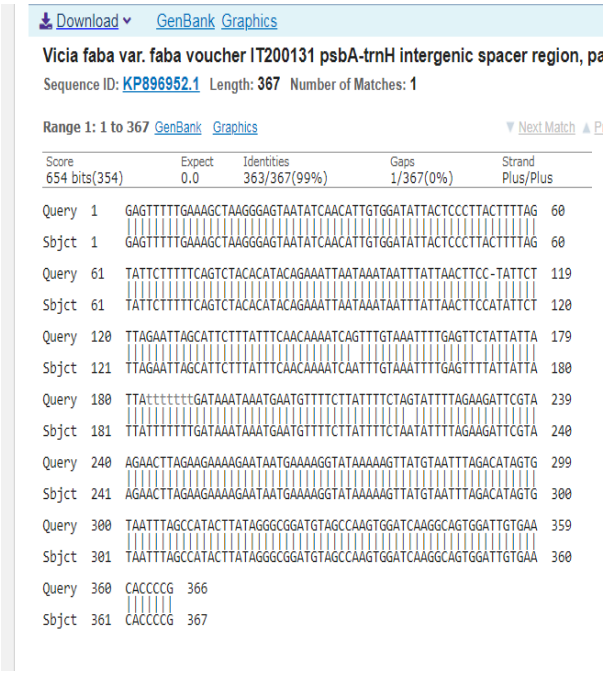
Fig. (3): region polymorphisms based on the number of bands with the use of the PCR-SSCP approach



Conditions for electrophoresis: 8% polyacrylamide gel concentration; run time 90 - 120 min, 200V (7.5V/cm) - 100mA. Ethidium bromide staining method

It could be challenging to determine the pattern regarding all resolved SSCP bands with the use of only gel visualization. Sequencing of DNA is after that required to confirm DNA polymorphisms. Sequence results show that various SNPs had identified ITS area haplotypes in accordance with NCBI Blast, as shown in Figure 4.

Fig. (4): Sequences alignment ID: KP896952.1 results for Vicia faba L Isolated From (nourth Of Country), ITS4 region by NCBI BLAST


Download GenBank Graphics

Vicia faba var. faba voucher IT200131 psbA-trnH intergenic spacer region, pa

Sequence ID: KP896952.1 Length: 367 Number of Matches: 1

Range 1: 1 to 367 GenBank Graphics Next Match

Score	Expect	Identities	Gaps	Strand
654 bits(354)	0.0	363/367(99%)	1/367(0%)	Plus/Plus

```
Query  1    GAGTTTTTGAAAGCTAAGGGAGTAATATCAACATTGTGGATATTACTCCCTTACTTTTAG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    GAGTTTTTGAAAGCTAAGGGAGTAATATCAACATTGTGGATATTACTCCCTTACTTTTAG  60

Query  61   TATTCTTTTTCAGTCTACACATACAGAAATTAATAAATAATTTATTAACTTCC-TATTCT  119
            ||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||
Sbjct  61   TATTCTTTTTCAGTCTACACATACAGAAATTAATAAATAATTTATTAACTTCCATATTCT  120

Query  120  TTAGAATTAGCATTCTTTATTTCAACAAAATCAGTTTGTAAATTTTGAGTTCTATTATTA  179
            ||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct  121  TTAGAATTAGCATTCTTTATTTCAACAAAATCAATTTGTAAATTTTGAGTTTTATTATTA  180

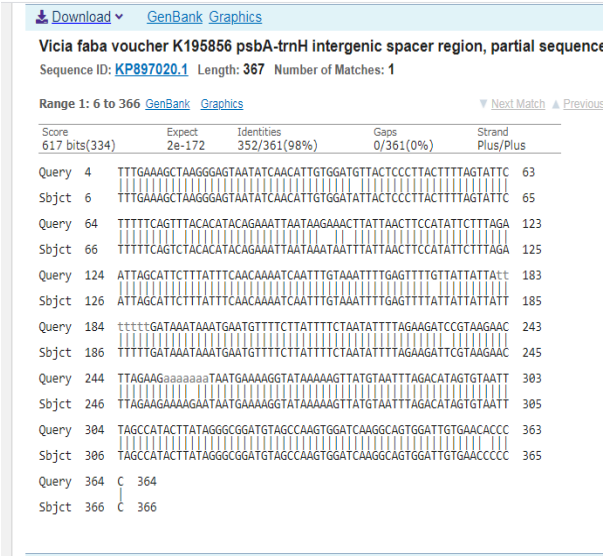
Query  180  TTAttttttGATAAATAAATGAATGTTTTCTTATTTTCTAGTATTTTAGAAGATTCGTA  239
            ||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  181  TTATTTTTTTGATAAATAAATGAATGTTTTCTTATTTTCTAATATTTTAGAAGATTCGTA  240

Query  240  AGAACTTAGAAGAAAAGAATAATGAAAAGGTATAAAAAGTTATGTAATTTAGACATAGTG  299
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  AGAACTTAGAAGAAAAGAATAATGAAAAGGTATAAAAAGTTATGTAATTTAGACATAGTG  300

Query  300  TAATTTAGCCATACTTATAGGGCGGATGTAGCCAAGTGGATCAAGGCAGTGGATTGTGAA  359
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  TAATTTAGCCATACTTATAGGGCGGATGTAGCCAAGTGGATCAAGGCAGTGGATTGTGAA  360

Query  360  CACCCCG  366
            |||||||
Sbjct  361  CACCCCG  367
```

Fig. (5): Sequences alignment ID: KP897020.1 results for Vicia faba L Isolated From (south Of Country), ITS4 region by NCBI BLAST


Download GenBank Graphics

Vicia faba voucher K195856 psbA-trnH intergenic spacer region, partial sequence

Sequence ID: KP897020.1 Length: 367 Number of Matches: 1

Range 1: 6 to 366 GenBank Graphics Next Match Previous

Score	Expect	Identities	Gaps	Strand
617 bits(334)	2e-172	352/361(98%)	0/361(0%)	Plus/Plus

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Query  4    TTTGAAAGCTAAGGGAGTAATATCAACATTGTGGATGTTACTCCCTTACTTTTAGTATTC  63
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6    TTTGAAAGCTAAGGGAGTAATATCAACATTGTGGATATTACTCCCTTACTTTTAGTATTC  65

Query  64   TTTTTCAGTTTACACATACAGAAATTAATAAGAAACTTATTAACTTCCATATTCTTTAGA  123
            |||||||||||||||||||||||||||| || ||||||||||||||||||||||||||||
Sbjct  66   TTTTTCAGTCTACACATACAGAAATTAATAAATAATTTATTAACTTCCATATTCTTTAGA  125

Query  124  ATTAGCATTCTTTATTTCAACAAAATCAATTTGTAAATTTTGAGTTTTGTTATTATTAtt  183
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  126  ATTAGCATTCTTTATTTCAACAAAATCAATTTGTAAATTTTGAGTTTTATTATTATTATT  185

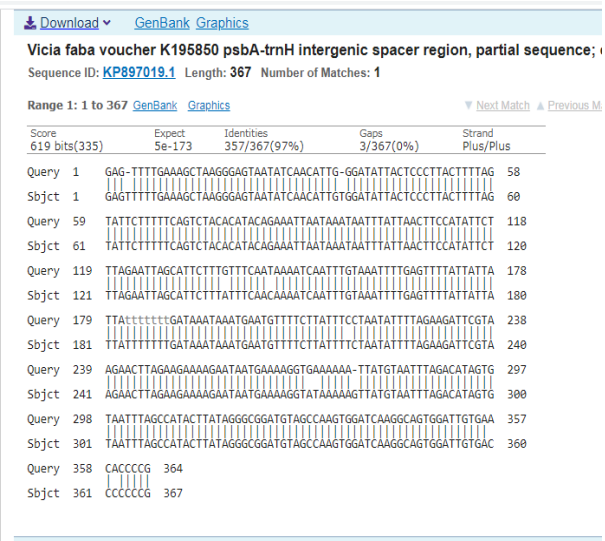
Query  184  ttttGATAAATAAATGAATGTTTTCTTATTTTCTAATATTTTAGAAGATCCGTAAGAAC  243
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  186  TTTTTGATAAATAAATGAATGTTTTCTTATTTTCTAATATTTTAGAAGATTCGTAAGAAC  245

Query  244  TTAGAAGaaaaaaaTAATGAAAAGGTATAAAAAGTTATGTAATTTAGACATAGTGTAATT  303
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  246  TTAGAAGAAAAGAATAATGAAAAGGTATAAAAAGTTATGTAATTTAGACATAGTGTAATT  305

Query  304  TAGCCATACTTATAGGGCGGATGTAGCCAAGTGGATCAAGGCAGTGGATTGTGAACACCC  363
            ||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||
Sbjct  306  TAGCCATACTTATAGGGCGGATGTAGCCAAGTGGATCAAGGCAGTGGATTGTGAACCCCC  365

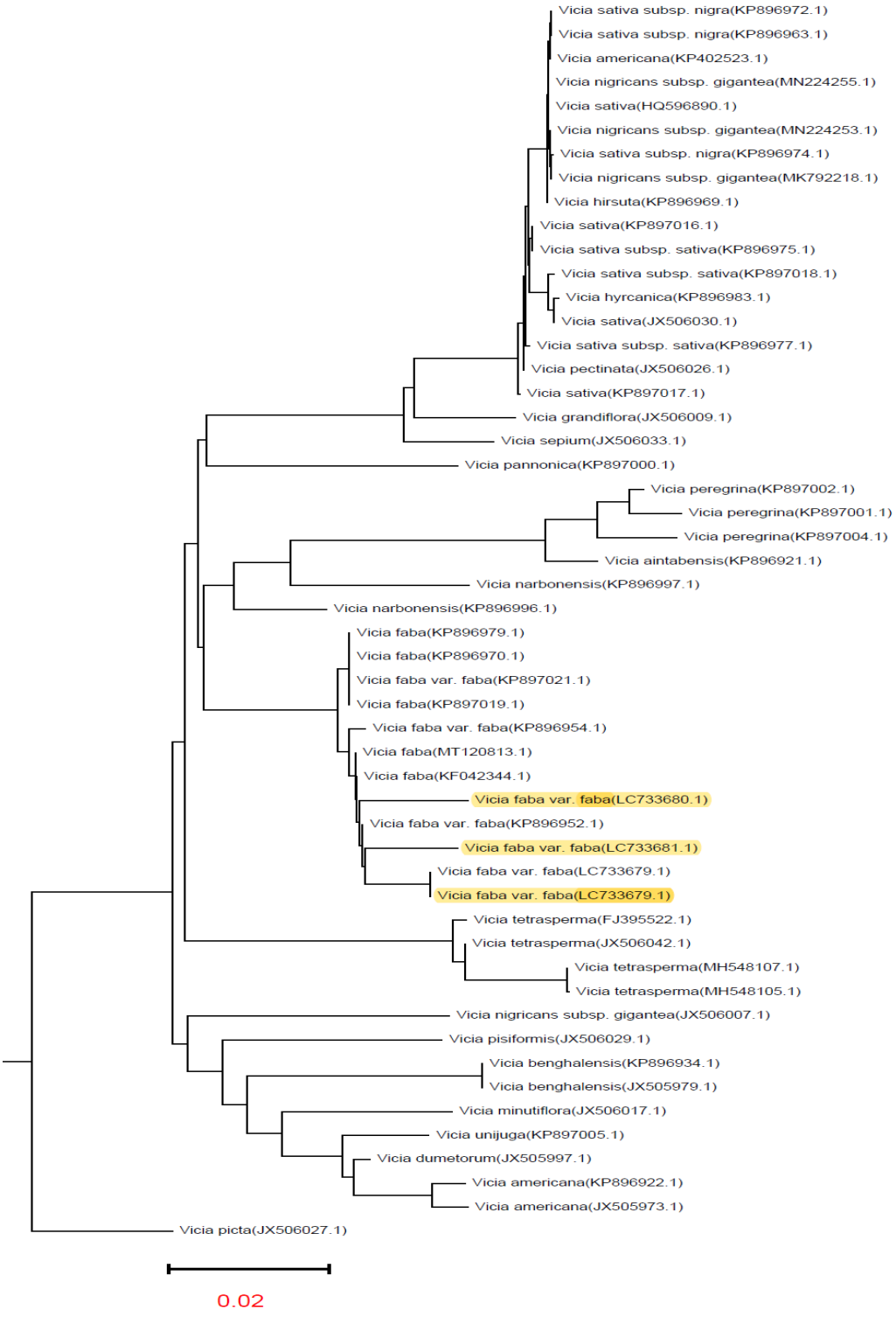
Query  364  C  364
            |
Sbjct  366  C  366
```

Fig. (6): Sequences alignment ID: KP897020.1 results for Vicia faba L Isolated From (middle Of Country), ITS4 region by NCBI BLAST



All 3 sequenced isolates were deposited in GenBank at DNA Data Bank Japan (DDBJ) database with accession numbers (LC733679 , LC733680, LC733681).

Fig. (7): phylogenetic tree anaylsis results for Vicia faba L, using by NCBI BLAST



Discussion

Due to the genus Vicia's economic significance, a significant amount of research was done on molecular characterisation and the study of phylogenetic relations among its species. These research projects used rdna (Ogihara and Raina,1995). Genetic diversity assessment has been given priority by plant curators, due to the fact that it is the initial step in creating global scenarios for issues like global warming, food safety, starvation, and climate changes. The barrier for marginal plants and therapeutic plants like fenugreek has increased since more services are being utilized for commercial or staple plants. It is necessary to use various biochemical and molecular approaches to identify various seed genotypes (Nikolic etal,,2016; Chandrawati etal,2016).

In the genus Vicia, Vicia is one of the biggest and most significant subgenera (Haider et al. 2000). There was a lot of investigation into this subgenus. Morphological analysis, RAPD, and cpDNA restriction utilizing PCR-RFLP, among other techniques (Jaaska and Leht 2002). Almost all mtDNA and nDNA-based investigations have been on V. faba, a significant food and feed legume due to high nutritional values of its seeds (van de Ven etal. 1993) (Duc etal. 2010).

Three unique haplotype models were identified by the number of stripes in PCR-SSCP gel electrophoresis findings, as illustrated in figure (3). According to Chalmers et al (2005) research's the ITS regions differ in series and size. Additionally, with the use of universally designed eukaryotic primers, the ITS region is easy to amplify from even herbary materials due to its modest size (>700 bp in angiosperms) and good retention of sequences (Hyvönen and Poczai, 2010).

Depending on nucleic acid's sensitivity to both the form and size of non-denaturing gel electrophoretic mobility, the SSCP theory was developed. dsDNAs have been transformed into ssDNAs through denaturation methods. SsDNAs, unlike the dsDNAs, are modular and adhere to conformation that is specific to the structure of their sequence that is dependent upon the intra-molecular interactions and fundamental folding. Variations in substitution sequences, like deletions and insertions in different electrophoretic movements, show that this ssDNA conformation could also affect the modification of one foundation (Doi etal,2004).

Biologists may now easily and affordably produce high-quality, large sequencing reads thanks to rapid technical advancements (Goodwin et al., 2016). The key variations between DNA platforms with regard to sequence identification recording techniques, amplification technology, and library production (Jian etal,2015).

DNA sequence homogeneity is needed in order to conduct molecular phylogenetic analysis. Phylogenetic analyses frequently use the internal ribosomal DNA transcribed spacer (ITS) area (nrDNA), although it is challenging to produce a homogenous sequence. For 9 pooled amplified ITS products, single-stranded conformation polymorphism (SAPS) technique had been employed in order to assess homogeneity. Our results demonstrate that the SSCP method was employed for detecting ITS homogeneity before it was used in sequencing operations (Hamidi etal,2014).

SSCP methodology, on the other hand, is a useful way for polymorphism identification. Since SSCP processes depend upon nucleotid heterogeneity regarding the haplotypes of the homologous sequences across 2 genomes, it also needs a high level of precision, and it isn't

ideal if we don't utilize the tool to identify similar genomic diversity estimation. In PCR amplified DNA, a single strand conformation polymorphism is a quick, reliable, and easy way to find insertions, deletions, and rearrangements (Guo etal,2012) (akseresht etal,2013).

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