Middle East Journal of Applied Sciences
Volume: 13 | Issue: 04| Oct. – Dec. | 2023

EISSN: 2706 -7947 ISSN: 2077- 4613
DOI: 10.36632/mejas/2023.13.4.55
Journal homepage: www.curresweb.com
Pages: 773-783

Genetic Diversity in Some *Vicia faba* L Cultivars by it DNA Barcode, (Scot) Polymorphic Markers and Protein Profile

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Received: 25 Sept. 2023 **Accepted:** 15 Nov. 2023 **Published:** 30 Dec. 2023

ABSTRACT

Faba bean (*Vicia faba* L.) is among the earliest domesticated legumes and remains a vital protein source for both human diets and animal feed, especially in the Middle East and North Africa. Despite its importance, the global cultivated area of this crop has gradually decreased without significant improvements in yield or stability. Limited availability of cultivars resistant to diseases and environmental stresses continues to restrict productivity. Understanding and exploiting genetic diversity is therefore essential for effective breeding strategies. In this work, seven Egyptian faba bean cultivars were evaluated using three complementary approaches: Internal Transcribed Spacer (ITS) DNA barcoding, Start Codon Targeted (SCoT) markers, and seed storage protein profiling. Analysis of polymorphic bands generated by SCoT primers, followed by cluster analysis, principal component analysis (PCA), and multivariate heatmap visualization, revealed three major cultivar groups corresponding to the Sakha, Nubaria, and Giza lines. The groupings were consistent with results obtained from ITS barcoding. Protein variation assessed through SDS-PAGE further confirmed the presence of intra-specific diversity. Overall, the study demonstrates substantial genetic differentiation among the examined cultivars and highlights valuable resources that can be exploited in future breeding programs aimed at enhancing yield and resilience.

Keywords: *Vicia faba* L., Start Codon Targeted (SCoT) markers, SDS-PAGE, DNA barcoding, ITS.

1. Introduction

Vicia faba L. is one of the oldest cultivated legumes and has long played a central role in agriculture and human diets. Its seeds provide a rich source of proteins, carbohydrates, fibers, and essential micronutrients, making it a valuable food in both developing and industrialized regions. Beyond its nutritional benefits, faba bean contributes to sustainable agriculture by fixing atmospheric nitrogen, enriching soil fertility, and enhancing productivity when incorporated into crop rotation or intercropping systems (Stagnari *et al*., 2017; O'Sullivan *et al*., 2016).

Despite its recognized importance, the crop still shows relatively modest yield potential roughly half that of wheat—which underlines the continuing need for focused breeding programs (FAO, 2017). Over recent decades, the global cultivation area of faba bean has gradually shrunk, while gains in productivity have remained limited. Key challenges include yield instability and the scarcity of cultivars with strong resistance to major pathogens.

Faba bean landraces and primitive lines are known to harbor significant genetic diversity, reflecting both natural adaptation to local conditions and selective pressures imposed by farming practices. DNA-based markers have shown that such diversity often correlates with ecological and geographical origins, offering opportunities to identify alleles valuable for breeding. Unlocking this variability is particularly critical in open-pollinated crops, where substantial genetic improvements can be realized.

To exploit this variation, researchers increasingly rely on biochemical and molecular marker systems. Compared with morphological traits, molecular markers provide higher resolution and are less influenced by environmental factors. Combining modern genomic approaches with classical

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breeding tools can accelerate the development of superior cultivars (Moose & Mumm, 2008). Such tools are useful for multiple objectives, including:

- Identifying favorable genes and alleles to improve selection efficiency (Collard *et al*., 2008);
- Evaluating gene action and breeding value through QTL mapping (Tamura *et al*., 2004); and
- Broadening the genetic base of cultivars by incorporating novel alleles using recombinant DNA technologies (Johnson *et al*., 2009).

The present study investigates the genetic diversity among seven Egyptian faba bean cultivars using three complementary methods: Start Codon Targeted (SCoT) markers, Internal Transcribed Spacer (ITS) DNA barcoding, and SDS-PAGE protein profiling. Together, these techniques provide insights into cultivar relationships, pedigrees, and potential applications in breeding programs

2. Materials and Methods

2.1. Plant Materials

Seven *Vicia faba* L cultivars were obtained from the Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center (ARC), Giza, Egypt. The selected cultivars are all of Egyptian origin and are listed along with their respective GenBank accession numbers for ITS gene sequences in Table 1.

Table 1: Origin and GenBank accession numbers for ITS sequences of the seven faba bean cultivars.

NO.	Origin	Cultivar name	ITS gene bank accession No
1	Egypt	Sakha1	MW386311
2	Egypt	Sakha3	MW465991
3	Egypt	Sakha4	MW463309
4	Egypt	Giza 3	MW463310
5	Egypt	Giza 40	MW463319
6	Egypt	Nubaria 2	MW463320
7	Egypt	Nubaria 3	MW463321

2.2. DNA Extraction, Purification, and Amplification

Genomic DNA was extracted from seedling leaves using the Thermo Scientific Gene JET Plant Mini Kit. DNA integrity and quality were confirmed by agarose gel electrophoresis, which revealed high molecular weight bands without degradation.

PCR amplification for ITS2 and SCoT markers was carried out in 20 µLtotal reaction volumes. For ITS2, the reaction mix contained: 10 µL of 2× PCR master mix, 1 µL of forward primer, 1 µL of reverse primer (10 pmol), 1 µL of genomic DNA, and 7 µL of sterile distilled water. For SCoT markers, the reaction mix included: 10 µL of 2× PCR master mix, 0.5 µL of each primer, 2 µL of genomic DNA, and 7.5 µL of sterile distilled water.
The PCR protocol was as follows:

- Initial denaturation at 94 °C for 3 minutes 37 cycles of:
  - Denaturation at 94 °C for 1 min.
  - Annealing at 56 °C (ITS2) or 50 °C (SCoT) for 30 sec.
  - Extension at 72 °C for 1 minute
  - Final extension at 72 °C for 10 min.

Amplicons were visualized on 1.5% agarose gels. Purified ITS2 PCR products were sequenced using an Applied Biosystems 3130 DNA Sequencer (ABI, Foster City, CA, USA).

The ITS region was amplified using universal primers ITS5 (5′-AAGGTTTCCGTAGGTGA AC-3′) and ITS4 (5′-TATGCTTAAACTCCAGCGGG-3′), as described by White *et al.* (1990). Sequence assembly was performed using BioEdit (v7.2.5), and sequence identity was confirmed via BLAST searches against public databases. Multiple sequence alignment was conducted using MEGA X (Kumar *et al.,* 2018), with gap refinement in Gblocks (Castresana, 2000; Talavera & Castresana,

2007). Phylogenetic trees were constructed using the UPGMA algorithm with 1,000 bootstrap replications, and node confidence values were calculated using SEQBOOT.

2.3. Protein Extraction and SDS-PAGE Analysis

Approximately 250 mg of seeds from each cultivar were ground in liquid nitrogen. From this, 100 mg of powdered seed flour was mixed with 500 µL of protein extraction buffer (10 mM HEPES, pH 7.5; 0.65 M NaCl; 1 mM EDTA; 0.34 M sucrose; and a protease inhibitor cocktail). Samples were homogenized using vortexing and centrifuged at 12,000×g for 5 minutes at room temperature.

The resulting supernatant (soluble proteins) was collected and stored at −20 °C for SDS-PAGE. Protein fractionation was performed using 10% resolving and 5% stacking polyacrylamide gels in a Mini-PROTEAN system (Laemmli, 1970). Electrophoresis was run at a constant voltage of 150 V in MOPS running buffer (50 mM MOPS, 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.7). A protein ladder with molecular weights ranging from 10–250 kDa was included as a reference.

After electrophoresis, gels were stained overnight with 0.25% Coomassie Brilliant Blue(COBB) R-250, followed by destaining in methanol/acetic acid for 45 minutes and further destained until the background was clear for scoring.

2.4 Data Analysis

Protein and SCoT electrophoretic banding patterns were analyzed by scoring bands as present (1) or absent (0). Similarity among cultivars was evaluated using Jaccard's similarity coefficient (Jaccard, 1908). Phylogenetic trees were generated using the UPGMA method via PAST software (Hammer *et al.,* 2001).

The discriminatory power of SCoT primers was evaluated by calculating:

- Polymorphic Information Content (PIC): According to Ghislain *et al.* (1999)
- Resolving Power (Rp): Calculated as $Rp = \Sigma IB$, where $IB = 1 - (2 \times |0.5 - p|)$ based on Gilbert *et al.* (1999)
- Effective Multiplex Ratio (EMR) and Marker Index (MI): As per Powell *et al.* (1996).

Multivariate analysis was conducted using heatmap clustering in R software (Berry *et al.,* 2006), and Principal Coordinates Analysis (PCoA) was applied to further assess genetic relationships among cultivars (Abbott, 2014).

3. Results

3.1 Molecular Characterization and Genetic Relationships Revealed by SCoT Markers

SCoT analysis using eight primers generated distinct amplification profiles across the seven faba bean cultivars (Figure 1). In total, 61 bands were obtained, with individual primers producing between 2 (SCoT 24) and 11 (SCoT 7, SCoT 9, and SCoT 46) fragments. With the exception of SCoT 24, which showed 50% polymorphism, all primers yielded fully polymorphic profiles, indicating considerable variation among the studied genotypes.

The polymorphic information content (PIC) ranged from 0.55 (SCoT 46) to 0.97 (SCoT 24), averaging 0.73, which confirms the high informativeness of these markers. The mean resolving power (Rp) was 2.80, and the corresponding values of marker index (MI), PIC, Rp, and EMR for each primer are summarized in Table 2.

Cluster analysis (UPGMA) grouped the cultivars into three major clusters (Figure 2).

- Cluster I separated into two subgroups: Giza 3 with Giza 40, and Nubaria 2 with Nubaria 3.
- Cluster II split into Sakha 3 and Sakha 1 as distinct branches.
- Cluster III consisted only of Sakha 4, highlighting its genetic distinctiveness.

Principal Component Analysis (PCA) further supported this grouping, producing three clear clusters consistent with the dendrogram (Figure 3). Likewise, multivariate heatmap analysis (Figure 4) confirmed the separation into three main groups, with Giza and Nubaria forming one cluster, while Sakha cultivars divided into two subclusters. Together, these results demonstrate the effectiveness of SCoT markers in capturing intra-specific genetic diversity.

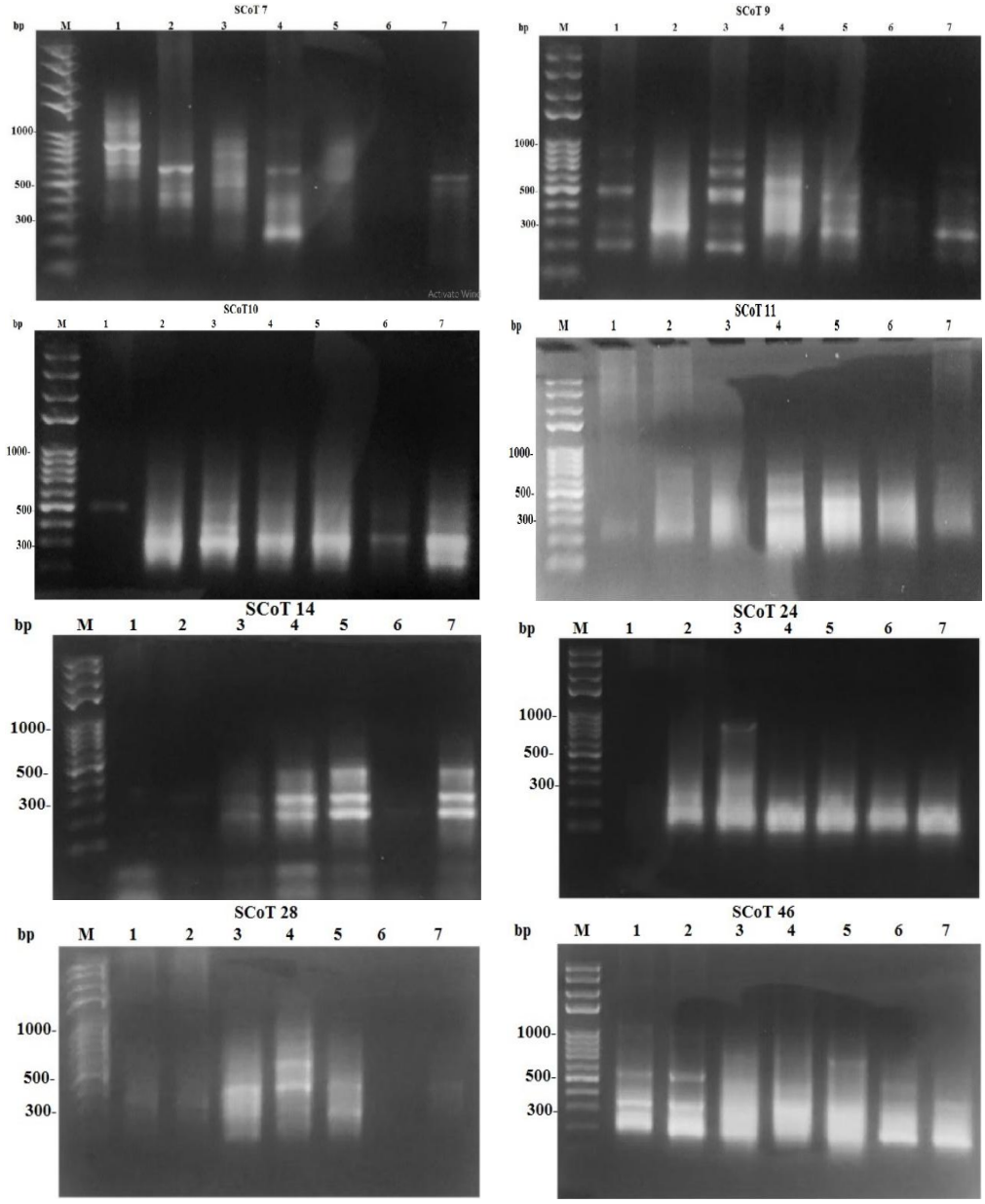

Fig. 1: Amplification profiles of genomic DNA from seven faba bean cultivars using eight different SCoT primers.

Lanes: M = DNA ladder; 1 = Sakha 1; 2 = Sakha 3; 3 = Sakha 4; 4 = Giza 3; 5 = Giza 40; 6 = Nubaria 2; 7 = Nubaria 3.

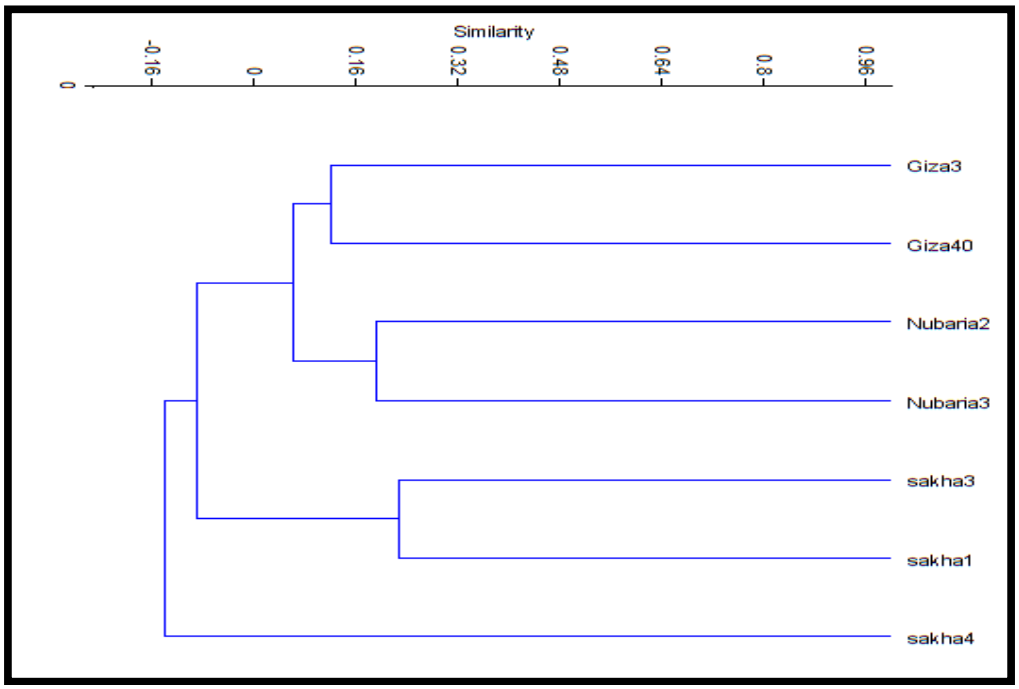

Fig. 2: Dendrogram representing the genetic relationships among seven faba bean cultivars based on Jaccard's similarity coefficients derived from eight SCoT markers, using the UPGMA clustering algorithm.

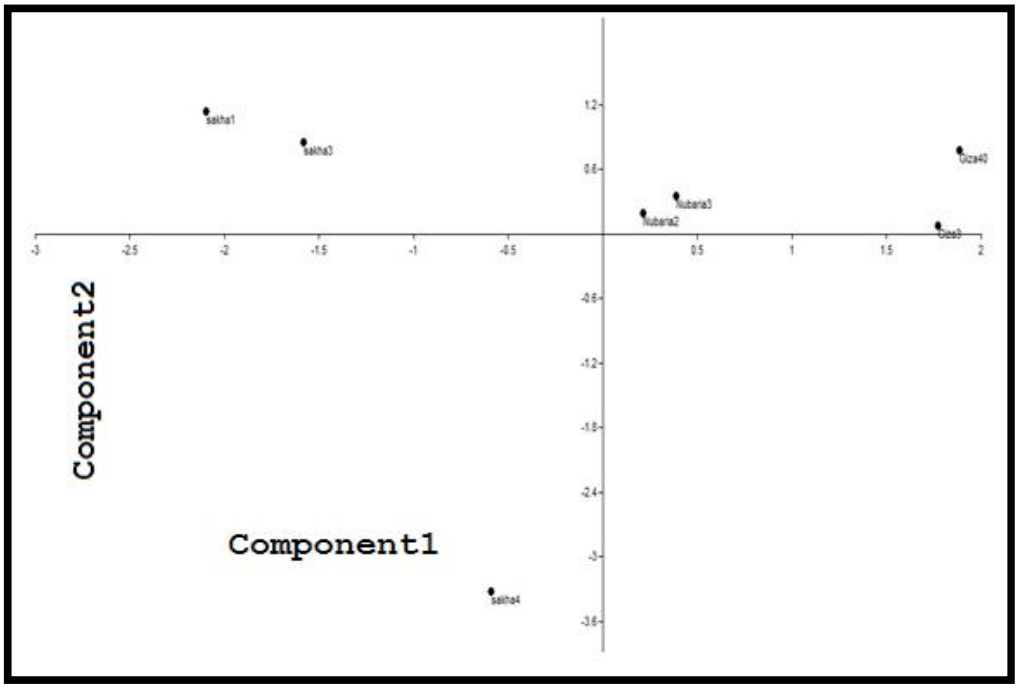

Fig. 3: Principal Component Analysis (PCA) plot illustrating the genetic relationships among seven *Vicia faba* cultivars based on SCoT marker data.

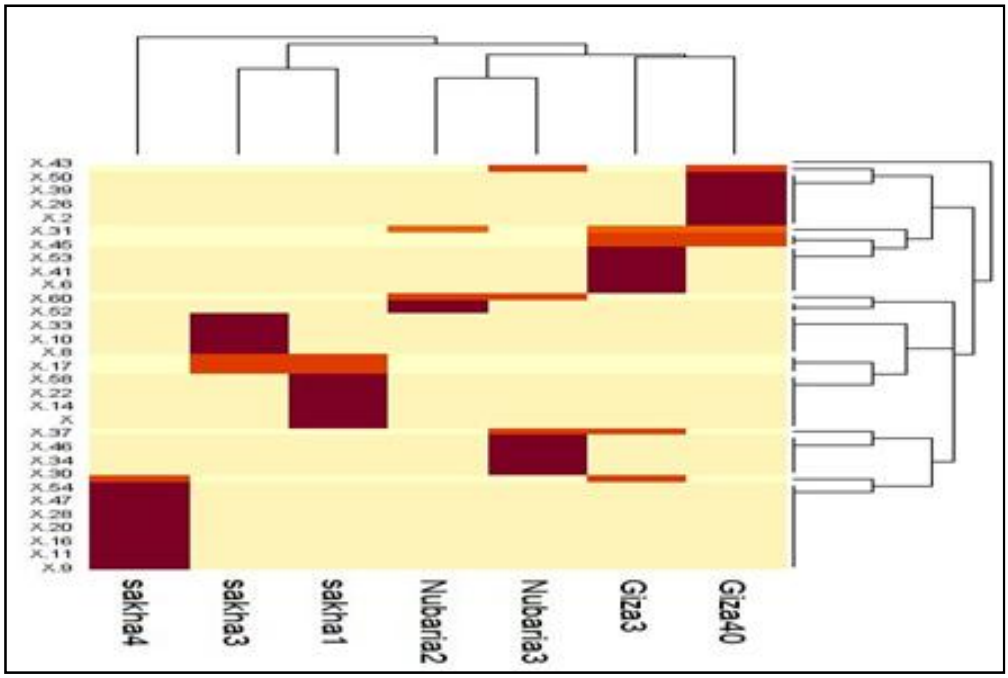

Fig. 4: Multivariate heatmap illustrating the genetic diversity among seven faba bean cultivars based on SCoT marker data, generated using the heatmap module in R software.

Table 2: Characteristics of SCoT primers used for genetic diversity analysis in seven faba bean cultivars. The table includes primer names and sequences, total number of bands (TNB), monomorphic bands (MB), polymorphic bands (PB), percentage of polymorphism (P%), polymorphism information content (PIC), resolving power (RP), effective multiplex ratio (EMR), and marker index (MI), as revealed by SCoT profiles.

SI No	Primer ID.	Sequence (5\' - 3\')	MBs	PBs	TNB	P%	MBF	PIC	EMR	RP	MI
1	Scot 7	ACAATGGCTACCACTGAC	0	11	11	100	0.16	0.70	11	3.44	7.70
2	Scot 9	ACAATGGCTACCACTGCC	0	11	11	100	0.17	0.64	11	3.74	7.04
3	Scot10	ACAATGGCTACCACCAGC	0	9	9	100	0.14	0.80	9	2.57	7.20
4	Scot 11	ACAATGGCTACCACTACC	0	3	3	100	0.24	0.77	3	3.43	2.31
5	Scot 14	ACCATGGCTACCAGCGCG	0	8	8	100	0.20	0.62	8	3.18	4.96
6	Scot 24	CCATGGCTACCACCGCAG	1	1	2	50	0.57	0.97	0.5	0.29	0.49
7	Scot 28	CAACAATGGCTACCACCA	0	6	6	100	0.17	0.80	6	2.02	4.80
8	Scot 46	ACCATGGCTACCACCGCC	0	11	11	100	0.19	0.55	11	4.03	6.05
	Total		1	60	61	NA	NA	NA	NA	NA	NA
	Mean			7.5	7,6	98.4	.23	0.73	16.80	2.80	4.76

3.2. DNA Barcoding of ITS Sequences

The ITS region was successfully amplified in all cultivars, producing clear, specific bands (Figure 5). Sequence analysis allowed deposition of all ITS accessions in GenBank (Table 1).

The UPGMA tree constructed from ITS sequences divided the cultivars into two major clades (Figure 6).

- The first clade comprised Nubaria 2 with Nubaria 3, and Giza 3 with Giza 40.
- The second clade contained Sakha 3 with Sakha 4, while Sakha 1 formed a separate branch.

Bootstrap support values (89–99%) confirmed the stability of these clusters, reflecting reliable phylogenetic relationships among the seven cultivars.

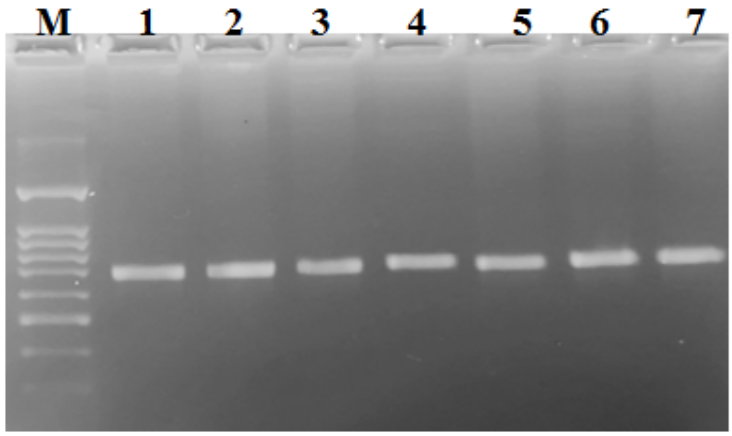

Fig. 5: Amplification profiles of genomic DNA from seven faba bean cultivars using ITS primers. Lanes: M = DNA ladder; 1 = Sakha 1; 2 = Sakha 3; 3 = Sakha 4; 4 = Giza 3; 5 = Giza 40; 6 = Nubaria 2; 7 = Nubaria 3.

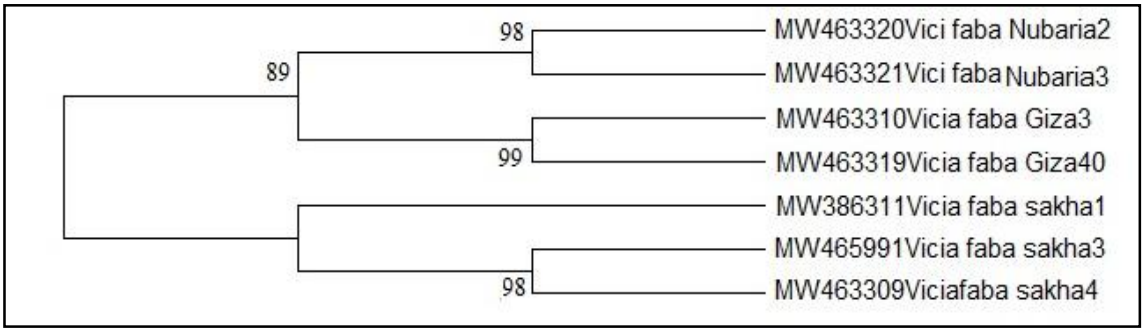

Fig. 6: Consensus phylogenetic tree illustrating the genetic relationships among seven *Vicia faba* cultivars based on ITS DNA barcoding sequences, constructed using the UPGMA algorithm in MEGA X software.

3.3. Protein Analysis

Protein profiling using SDS-PAGE detected 29 bands ranging in molecular weight from 22.5 to 112.02 kDa, of which 22 were polymorphic (78.57%). Each cultivar showed a distinct pattern, allowing differentiation based on seed protein composition (Figure 7).

UPGMA clustering based on Nei's genetic distance (Nei, 1972) grouped the cultivars into two main clusters (Figure 8):

- Cluster I consisted solely of Nubaria 3, reflecting its unique protein profile.
- Cluster II separated into three subgroups: (i) Giza 40 with Sakha 1, (ii) Sakha 3 with Nubaria 2 and Sakha 4, and (iii) Giza 3 as a distinct subgroup.

These results demonstrate that SDS-PAGE protein profiling, although less detailed than DNA-based markers, provides useful complementary information for distinguishing cultivars and assessing intra-specific variation.

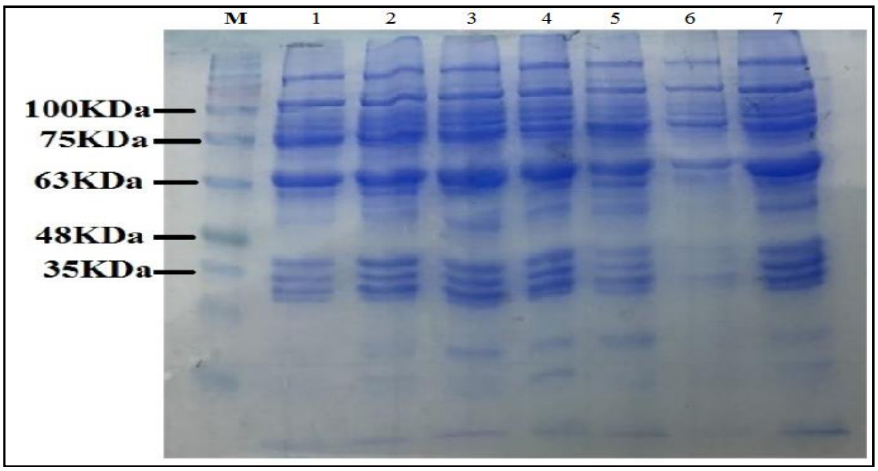

Fig. 7: SDS-PAGE protein profiles of seven faba bean cultivars.
Lanes: M = protein molecular weight marker; 1 = Sakha 1; 2 = Sakha 3; 3 = Sakha 4; 4 = Giza 3; 5 = Giza 40; 6 = Nubaria 2; 7 = Nubaria 3.

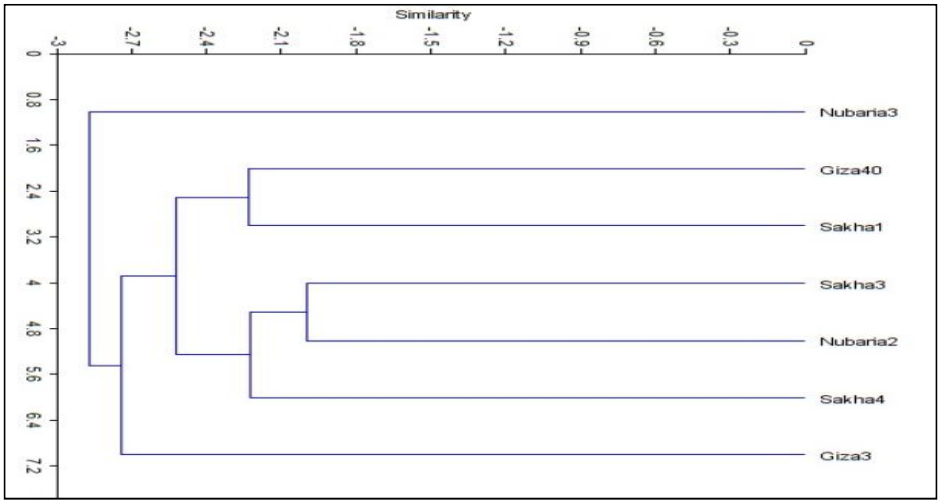

Fig. 8: Dendrogram illustrating the genetic similarity among seven faba bean cultivars, constructed using the UPGMA clustering method based on Jaccard's similarity coefficients derived from SDS-PAGE protein banding patterns.

4. Discussion

The worldwide area devoted to faba bean cultivation has slightly declined in recent years, although overall yields have remained fairly stable. This trend is often linked to the limited resistance of existing cultivars to diseases and abiotic stresses, as well as fluctuations in yield stability. To overcome these challenges, breeding efforts need to focus on developing new cultivars with improved tolerance and adaptability.

In this study, three complementary approachesSCoT markers, ITS DNA barcoding, and SDS-PAGE protein profiling—were employed to examine the genetic variability among seven Egyptian *Vicia faba* cultivars. Each technique offered distinct insights into the genetic structure and relationships of the tested genotypes.

SCoT markers, which are particularly effective in detecting low-level polymorphism (Collard & Mackill, 2009), produced highly informative results. The eight primers generated 61 bands, averaging 7.6 per primer, with a polymorphism rate close to 98.4%. Except for one primer, all revealed complete polymorphism. The overall PIC value (0.73) demonstrated strong discriminatory ability, comparable to earlier findings in legume and cereal studies (Monier *et al*., 2021; Nosair, 2020; Zeid *et al*., 2009). Additional indices such as resolving power (Rp) and marker index (MI) also confirmed the efficiency of SCoT primers, in line with previous reports (Prevost & Wilkinson, 1999; Smith *et al*., 1997).

Multivariate analyses, including UPGMA clustering, PCA, and heatmap visualization, consistently identified three cultivar groups. Notably, Sakha 4 remained genetically distinct, whereas Sakha 1 with Sakha 3, Nubaria 2 with Nubaria 3, and Giza 3 with Giza 40 formed separate subclusters.

The ITS region, a widely accepted DNA barcode in plants (Alvarez & Wendel, 2003), provided additional support for these groupings. As a relatively fast-evolving marker, ITS sequences are suitable for both interspecific and intraspecific comparisons (Jorgenson & Cluster, 1988; Baura *et al*., 1992). In the present study, ITS-based phylogeny divided the cultivars into two main clades, broadly consistent with the SCoT-derived relationships. The clustering of the Sakha cultivars together and the grouping of Nubaria and Giza cultivars into distinct branches, supported by high bootstrap values (89–99%), reinforces the reliability of the ITS results (Figure 6).

Protein profiling via SDS-PAGE added another layer of differentiation. Out of 29 observed bands, 22 were polymorphic, representing 78.57% variation. Such results agree with earlier studies that showed the usefulness of seed storage proteins as biochemical markers for cultivar identification (Iqbal *et al*., 2005; Hameed *et al*., 2014). The clustering pattern based on protein profiles partially overlapped with molecular results: Nubaria 3 appeared as an independent group, Nubaria 2, Sakha 3, and Sakha 4 clustered together, and Giza 3 separated into its own subgroup.

Interestingly, the correlation among the three methods (SCoT, ITS, and SDS-PAGE) was relatively weak. Similar outcomes have been reported previously (Najla Al Shaye *et al*., 2018), reflecting the fact that each system captures variation at different biological levels: DNA markers show polymorphism in genomic sequences, ITS evaluates ribosomal regions, and protein markers reveal expressed gene products. While molecular markers highlight genotypic diversity, protein-based methods detect differences in gene expression, and the two are not always directly correlated (Khan *et al*., 2009).

This observation underscores the importance of integrating multiple approaches when assessing genetic variation. Combining molecular and biochemical data provides a more comprehensive view of diversity, which is essential for breeding applications (Pandey *et al*., 2015; Parsaeian *et al*., 2011). Since morphological and molecular traits can diverge due to genetic complexity and environmental influences (Tabatabaei *et al*., 2011), SDS-PAGE remains a valuable complement to DNA-based tools, despite its sensitivity to environmental conditions.

4. Conclusion

This investigation revealed clear genetic variation among the seven Egyptian *Vicia faba* cultivars when analyzed with SCoT markers, ITS DNA barcoding, and SDS-PAGE protein profiling. The combined use of these molecular and biochemical techniques provided consistent evidence of intra-specific diversity and proved effective in distinguishing between the studied genotypes.

The identified variability represents an important genetic reservoir that breeders can utilize for developing improved cultivars with better yield potential, stress resistance, and adaptability. To maximize these benefits, future breeding programs should place greater emphasis on systematic collection, conservation, and comprehensive characterization of faba bean genetic resources. Such efforts will expand the genetic base available for crop improvement and facilitate the introduction of cultivars that are more resilient and productive under diverse agricultural conditions.

5. Acknowledgment

The author would like to express sincere gratitude to Prof. Dr. Mohamed Mohamadein Mokhtar, Professor of Microbiology and Biology at the Regional Center for Mycology and Biotechnology, Al-Azhar University, for his valuable guidance and support throughout the course of this study.

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