Novel Research in Microbiology Journal (2024), 8(2): 2370-2392

(Print) (ISSN 2537-0286)
(Online) (ISSN 2537-0294)
https://nrmj.journals.ekb.eg/

Research Article

DOI: 10.21608/NRMJ.2024.276809.1495

Evaluation of genetic diversity of some *Zucchini yellow mosaic virus*-infected cucurbits based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, inter simple sequence repeat and start codon targeted molecular tools

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Received: 14 March, 2024; Accepted: 15 April, 2024; Published online: 16 April, 2024

Abstract

The aim of this study was to evaluate the genetic diversity of some *Zucchini yellow mosaic virus* (ZYMV)-infected cucurbits compared to the healthy plants based on three molecular tools. When sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was used to determine the genetic variability of four cucurbits infected with ZYMV compared to the healthy ones, a total of 15 storable protein bands were recorded. The experimental results showed that DNA polymorphisms included 38 polymorphic DNA fragments that were generated using the five inter simple sequence repeat (ISSR-PCR) used primers. The maximum identity (%) among the ZYMV-infected and healthy samples was 59, 56, 45, 40, 30, and 29 % for the plant samples of watermelon, squash, cantaloupe, qethaa, cucumber, and luffa samples, respectively. Phylogenetic tree of DNA polymorphisms confirmed the genetic relationship between each of the healthy samples of watermelon and cucumber, healthy samples of qethaa and cucumber, ZYMV-infected samples of watermelon and squash, and ZYMV-infected samples of kantaloupe and qethaa. Numbers of nine start codon targeted (SCoT) primers were used to determine the DNA fingerprinting of six ZYM-infected cucurbit species compared to the healthy plants of the same species. A total of 88 polymorphic DNA fragments were distributed as follows: 13, 7, 10, 6, 9, 10, 15, 8, and 10, which were generated using SCoT-02, SCoT-03, SCoT-04, SCoT-06, SCoT-08, SCoT-11, SCoT-12, SCoT-13, and SCoT-14 primers, respectively. The DNA polymorphisms of the six ZYMV-infected cucurbit species showed identities that ranged from 32 to 79 % using nine SCoT primers compared to the healthy species.

Keywords: Genetic diversity, *Zucchini yellow mosaic virus*, Cucurbits, SDS-PAGE, ISSR-PCR, SCoT

1. Introduction

Zucchini yellow mosaic virus (ZYMV) is a member of the genus *Potyvirus* of family *Potyviridae*, which is considered as one of the most series viruses affecting the cucurbit crops (Ashfaq *et al*., 2021; Ahsan *et al*., 2023). Several sever systemic symptoms have been recorded in ZYMV-infected cucurbits; especially in the squash (*Cucurbita pepo* L.) plants whatever under field or greenhouse conditions, including blisters, vein banding, mosaic, yellows, and leaves deformation that has been ended by the formation of filiform shape leaves (Hammad *et al*., 2022; Ali *et al*., 2023).

The genetic diversities or genetic relationships among the cucurbit plants were determined using different molecular marker techniques, mainly random amplified polymorphic DNA (RAPD) (Mahfouzea *et al*., 2018), inter simple sequence repeat (ISSR) (Dje *et al*., 2010), simple sequence repeats (SSRs) (Barzegar *et al*., 2013), start codon targeted (SCoT) polymorphic markers (Mondal and Bandyopadhyay, 2024), conserved DNA derived polymorphism (CDDP) (Hajibarat *et al*., 2015), amplified fragment length polymorphisms (AFLPs) (Kim *et al*., 2021), restriction fragment length polymorphisms (RFLPs) (Sheeja *et al*., 2021), and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Rayan and Osman, 2019). Molecular DNA markers such as RAPD, ISSR and SSR were successfully used to determine the genetic diversity of the different carnation genotypes for designing future breeding strategies and mutants (Sharma *et al*., 2022).

The advantages of SSR and RAPD can be summarized in being high polymorphic, can distinguish among the heterozygotes, have high level of polymorphism, and are reliable tools (Dje *et al*., 2010). The ISSR and SCoT markers have been successfully applied to evaluate the genetic diversity of different plants including Gandhi, a threatened medicinal cucurbit (Gupta *et al*., 2021; Kulyan *et al*., 2023).

Borges and de Melo, (2019) estimated the genetic diversity among 12 pumpkin genotypes using ISSR molecular markers by amplifying 2671 DNA fragments that were analyzed by un-weighted pair group method with arithmetic (UPGMA) clustering. The results showed different similarities of 49.73, 45.74, 36.24, and 31.72 % among the clusters and confirmed the genetic variability among the evaluated genotypes. A previous study reported by Sevindik *et al*., (2020) used fifteen ISSR primers in ISSR markers to study the genetic diversity of some Turkish *Prunus armeniaca* L. genotypes obtained from Malatya province.

Guo *et al*., (2012) used SCoT polymorphic markers to determine the genetic relationships among 64 grape varieties using 36 primers. Their results showed that 17 primers producing 131 clear and repeatable polymorphic DNA fragments among the varieties had been selected. They added that the SCoT markers were informative and could be applied to detect polymorphism for the grape varieties. Rayan and Osman, (2019) used SCoT-PCR and electrophoretic SDS-PAGE techniques to determine the DNA-fingerprints and genetic diversity of six Egyptian soybean (*Glycine max* L.) genotypes. Eleven primers of SCoT technique were applied to generate the reproducible DNA polymorphic products, which were totally 106 classified into 52 and 54 polymorphic and monomorphic fragments, respectively. A total number of 23 bands were revealed via SDS-PAGE with a polymorphism of 30.43 %. For SDS-PAGE, the highest similarity index ranged from 0.714 to 0.909 among the tested soybean genotypes. The objective of this study was to evaluate the genetic diversity of some ZYMV-infected cucurbits compared to the healthy

plants based on SDS-PAGE, ISSR, and SCoT molecular markers.

2. Materials and methods

2.1. Source of cucurbit seeds and ZYMV isolate

Seeds of six cucurbit cultivars named Squash *cv*. Eskandarani, kantaloupe, watermelon, cucumber, qethaa, and luffa were kindly provided by the Institute of Horticultural Research, Agricultural Research Center (ARC), Giza, Egypt, and were cultivated under greenhouse conditions at the Laboratory of Virology, Department of Agricultural Microbiology, Faculty of Agriculture, Ain Shams University, Cairo, Egypt. Four seeds were individually cultivated in a 10 cm-plastic pots containing steam sterilized soils (370-380 g/ pot). The infectious viral sap was prepared in 0.01M phosphate buffer, pH 7.0. A weight of 3 g of ZYMV-infected squash leaves that exhibited the characteristic symptoms of ZYMV was prepared. The leaves were cut into small pieces using a sterile scalpel, placed in a sterile Chinese mortar, 6.0 ml of 0.01M sodium phosphate buffer (pH 7.0) were added followed by mashing with the buffer solution, and then filtering the sap using a sterile gauze. Finally, a filtered sap containing the virus was obtained.

2.2. Inoculation of the tested cucurbit species

The cotyledons of cucurbit plant species with 3-4 old leaves were mechanically inoculated with the ZYMV-infectious sap according to the method adopted by Spadotti *et al*., (2015) in the presence of carborandum (400 mesh) as an abrasive material. Groups of the same cucurbit species were left without ZYMV-inoculation to serve as controls.

2.3. Genetic variation of some ZYMV-infected cucurbit species

2.3.1. Based on protein patterns *via* SDS-PAGE analysis

Using SDS-PAGE protocol of Laemmli, (1970), the protein fingerprinting was conducted for ZYMV-infected cucurbit plant species 10 d post inoculation compared to the healthy plants of the same age. The protein sample was mixed with 2X SDS-PAGE sample buffer (1:1, v:v), heated in a water bath for 2 min. at 95oC to denature the proteins, and cooled in ice. The sample was loaded and then run was done at 90 V at room temperature for 16 h. The gel was then stained for overnight in Coomassie Brilliant blue followed by distaining to visualize the protein pattern. The gel was analyzed to determine the protein patterns of each sample as follows: clear and distinct protein bands were recorded as (1) for a present band and (0) for an absent band for all the infected plant samples. The similarity matrix coefficients were calculated among the different genotypes using the Nei and Li genetic similarity index formula reported by Sheet *et al*., (2018):

$$SI = 2Nij/Ni + Nj$$

Where: SI: Similarity index, Nij: the number of common bands shared between genotype (i) and genotype (j); Ni and Nj: the total number of DNA bands for genotypes. This matrix was used to construct a phylogenetic tree (Dendrogram).

2.3.2. Based on DNA *via* ISSR and SCoT

The total DNA was extracted and purified using the DNeasy Plant Mini Kit (Qiagen Santa Clarita, CA), following the manufacturer's instructions that were reported by Abdel Razek *et al*., (2022). Concentration of the genomic DNA was estimated using the NanoDrop to adjust its concentration at 50 ng/ µl, thus could be used as a template in PCR amplification (Desjardins and Conklin, 2010). Five inter simple sequence repeat-polymerase chain reaction primers [ISSR- 3, (5'- (AC)8T -3'); ISSR- 4 (5'-(AC)8G -3'); ISSR -6 (5'- (CG)2AT(AGAT)3A-3'); ISSR- 8 (5'-(AGAC)4GC-3'), and ISSR-10 (5'-(5'-(AGAC)4AT-3')] were used for the determining the DNA polymorphisms of the six cucurbits infected with ZYMV compared to the healthy plants of the same species. According to the previous study conducted by Abouseada *et al*., (2023), a mixture of 25 µl was prepared, which consisted of 12.5 µl Master Mix (sigma), 2.5 µl primer (10 pmol), 3.0 µl template DNA

(50 ng), and 7.0 µl d.H2O. PCR amplification was conducted in a Perkin-Elmer/ GeneAmp® PCR System 9700 (PE Applied Bio-systems) programmed among 40 cycles after an initial denaturation cycle for 5 min. at 94 ºC. Each cycle included a denaturation temperature of 94 ºC for 1 min., annealing temperature of 45 ºC for 1 min., and extension temperature of 72 ºC for 2 min. The final temperature (72 ºC) was extended to another 7.0 min. In this assay, to evaluate the genetic diversity among the ZYMV-infected and healthy cucurbits, nine SCoT primers were applied for generation of DNA polymorphisms of the DNA templates that were prepared from the six ZYMV-infected cucurbits; with the nucleotide sequences of SCoT-2 (5'-ACCATGGCTACCACCGGC-3'), SCoT-3 (5'-ACG ACA TGG CGA CCC ACA-3'), SCoT-4 (5'-ACC ATG GCT ACC ACC GCA-3'), SCoT-6 (5'-CAA TGG CTA CCA CTA CAG-3'), SCoT-8 (5'-ACA ATG GCT ACC ACT GCC-3'), SCoT-11 (5'-ACA ATG GCT ACC ACC AGC-3'), SCoT-12 (5'-CAA CAA TGG CTA CCA CCG-3'), SCoT-13 (5'-ACC ATG GCT ACC ACG GCA-3'), and SCoT-14 (5'-ACC ATG GCT ACC AGC GCG-3'). PCR mixture was prepared exactly as mentioned in ISSR-PCR analysis using the SCoT primers according to Abouseada *et al*., (2023). Similar PCR program was conducted in the same PCR thermo cycler machine with slight differences in the length of the 40 cycles, which were replaced with 45 sec., 50 sec., and 1.0 min. for the denaturation, annealing, and elongation temperatures, respectively.

2.3.3. Resolving the DNA polymorphisms of ISSR-PCR and SCoT markers

DNA polymorphisms generated via ISSR-PCR or SCoT marker methods were resolved using electrophoresis (Bogiel *et al*., 2022) in a 1.5 % agarose gel stained with ethidium bromide (0.5 µg/ ml) in 1X TBE buffer at 100 volts. PCR products were visualized using UV light illuminator and photographed using a Gel Documentation System (BIO-RAD 2000).

2.3.4. Data analysis of ISSR-PCR and SCoT markers

The generated DNA polymorphisms were visually scored as either present (1) or absent (0) for all the tested samples, and the final data sets included both of the monomorphic and polymorphic DNA fragments. Then, a binary statistic matrix was constructed. Dice's similarity matrix coefficients were then calculated among the different genotypes using the un-weighted pair group method with arithmetic averages (UPGMA) to construct a phylogenetic tree (Dendrogram) using the PAST software Version 1.91, according to the Euclidean similarity index (Hammer *et al*., 2001).

3. Results

3.1. Genetic diversity of ZYMV-infected cucurbits using SDS-PAGE analysis

In this study, SDS-PAGE analysis was used to determine the genetic variability of four cucurbits; mainly qethaa (Q), luffa (L), squash (S), and kantaloupe (K) infected (I) with ZYMV compared to the healthy (H) as shown in Fig. (1). Scoring of protein patterns of the four cucurbits whatever ZYMV-infected or healthy ones showed the presence of a total of 15 scorable protein bands. These bands were differed among the four cucurbits, where 6, 6, 7, 8, 8, 10, 11, and 13 bands were scored for KH, KI, LH, QH, QI, LI, SH, and SI, respectively. It was noted that the ZYMV-infected and healthy plants of qethaa and kantaloupe showed the same number of bands (*i.e*., 8 and 6, respectively). On the other hand, ZYMV-squash (13 and 10) and luffa plant samples generated a number of bands higher than the healthy ones, recording 11 and 7, respectively. Results presented in Table (1) showed the similarity % of protein profiles of the four cucurbits infected with ZYMV compared to the healthy plants based on SDS-PAGE analysis. The % protein profile of the ZYMV-infected and the healthy plants for each cucurbit were 53, 67, 83, and 83 % for luffa, qethaa, cantaloupe, and squash, respectively. Similarities % among the healthy cucurbits ranged from 53 to 82 %, while a range of 43-71 % was recorded among the ZYMV-infected cucurbits.

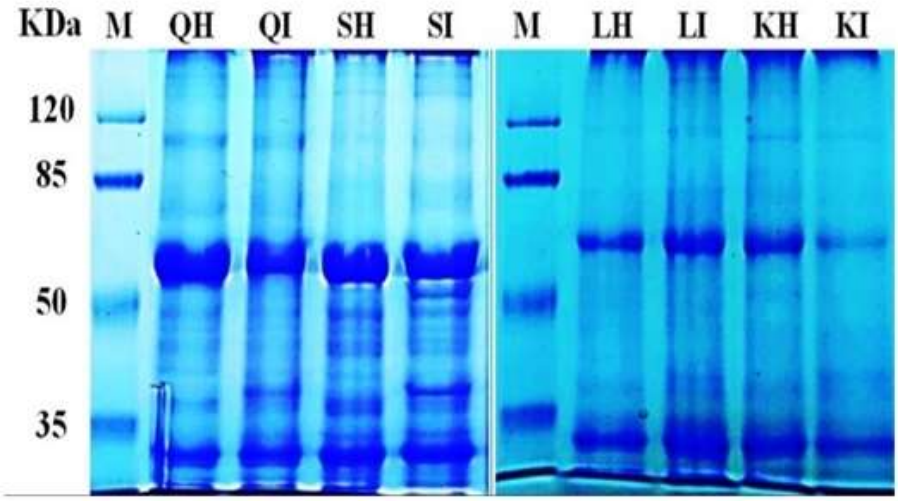

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of protein patterns of four *Zucchini yellow mosaic virus* (ZYMV) infected cucurbit species compared to the healthy ones. Where; QH: Qethaa healthy; QI: Qethaa infected; SH: Squash healthy; SI: Squash infected; LH: Luffa healthy; LI: Luffa infected; KH: Kantaloupe healthy; KI: Kantaloupe infected

Table 1: Identities (%) of the protein profiles of some cucurbits plants infected with *Zucchini yellow mosaic virus* (ZYMV) compared to the healthy ones based on Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Plant samples	**% identity of the protein profile**							
	QH	QI	SH	SI	LH	LI	KH	KI
QH	100							
QI	67	100						
SH	76	63	100					
SI	70	57	83	100				
LH	82	53	78	60	100			
LI	67	50	63	67	53	100		
KH	63	57	59	53	77	57	100	
KI	63	43	71	53	77	71	83	100

Where; QH: Qethaa healthy; QI: Qethaa infected; SH: Squash healthy; SI: Squash infected; LH: Luffa healthy; LI: Luffa infected; KH: Kantaloupe healthy; KI: Kantaloupe infected

A phylogenetic tree of the protein profiles of the four cucurbits (ZYMV-infected and healthy) based on SDS-PAGE analysis is shown in Fig. (2). Results revealed that the ZYMV-infected plants and the healthy ones from the same cucurbit species were located together in the same sub-cluster, *i.e*., the healthy and infected plants of each of luffa (LH and LI), kantaloupe (KH and KI), and squash (SH and SI). On the contrary, the samples of qethaa (ZYMV-infected and healthy) were located into two separate clusters. Based on these findings, the SDA-PAGE analysis proved to be suitable for differentiation among the different cucurbits whatever they were healthy or ZYMV-infected.

3.2. ISSR-PCR

The experimental results demonstrated in Fig. (3) and presented in Table (2) showed that the DNA polymorphisms included total amplified DNA fragments of 10, 7, 7, 7 and, 7, which were generated using the ISSR-03, ISSR-04, ISSR-06, ISSR-08, and ISSR-10 primers; respectively, upon using the total DNA extracts of the twelve cucurbits (six infected with ZYMV and six healthy) as templates in the PCR reactions. Meanwhile, a number of 38 DNA fragments were recorded among ISSR assessment of the genetic variations of the ZYMV-infected plants compared to the healthy ones of the same species. It was noted that the samples of squash, kantaloupe, qethaa, watermelon, cucumber, and luffa appeared on an average of 12.5, 20, 15.5, 15.5, 13.5, and 10.5 DNA fragments. The results also revealed that the healthy samples of each of squash, cantaloupe, and cucumber appeared in higher numbers of DNA fragments than the ZYMV-infected samples. On the contrary, the ZYMV-infected samples of qethaa, watermelon, and luffa were higher than the healthy ones. It is worth mentioning that all DNA fragments were of polymorphic types and no unique DNA markers were recorded. In other mean, no monomorphic DNA fragments were generated using the five ISSR primers. Data shown in Table (3) represent the identity percentages of DNA polymorphisms of the six cucurbit species infected with ZYMV compared to the healthy ones using five ISSR primers. Results showed that the maximum identity % among the ZYMV-infected and healthy plant samples was 59 % among the samples of watermelon, 56 % for squash, 45 % for kantaloupe, 40 % for qethaa, 30 % for cucumber, and 29 % for luffa samples. When the healthy plant samples were compared to each other among the six species of cucurbits, a range of identity ranging from 26 % (QH) to 77 % (WH) was recorded, where the lowest percent (26 %) existed between CI and LH samples, while the highest percent (77 %) was observed between watermelon and cucumber. Based on these findings, it could be suggested that, watermelon was genetically related to cucumber (77 %) and squash (64 %); while kantaloupe was related to cucumber (65 %), squash (61 %), and qethaa (50 %). Regarding the ZYMV-infected samples, the kantaloupe samples could be genetically related to qethaa (74 %), cucumber (67 %) and watermelon (56 %). A phylogenetic tree of DNA polymorphisms of the six cucurbit species infected with ZYMV compared to the healthy ones using five ISSR primers (Fig. 4) confirmed the genetic relationship among each of the healthy samples of watermelon and cucumber, and the healthy samples of qethaa and cucumber; ZYMV-infected samples of watermelon and squash, and ZYMV-infected samples of kantaloupe and qethaa.

3.3. SCoT method

In this investigation, nine SCoT primers were used among SCoT tool to determine the DNA fingerprinting of the six ZYM-infected cucurbits compared to the healthy plants of the same species. Results demonstrated in Fig. (5) and presented in Tables (4) and (5) showed the total amplified fragments of DNA polymorphisms of the tested samples.

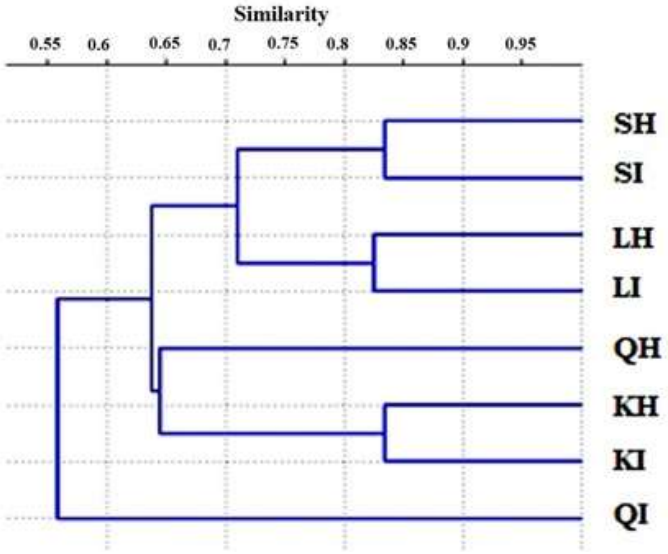

Fig. 2. A phylogenetic tree of protein profiles of some cucurbits infected with *Zucchini yellow mosaic virus* (ZYMV) compared to the healthy ones based on Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Where; QH: Qethaa healthy; QI: Qethaa infected; SH: Squash healthy; SI: Squash infected; LH: Luffa healthy; LI: Luffa infected; KH: Kantaloupe healthy; KI: Kantaloupe infected

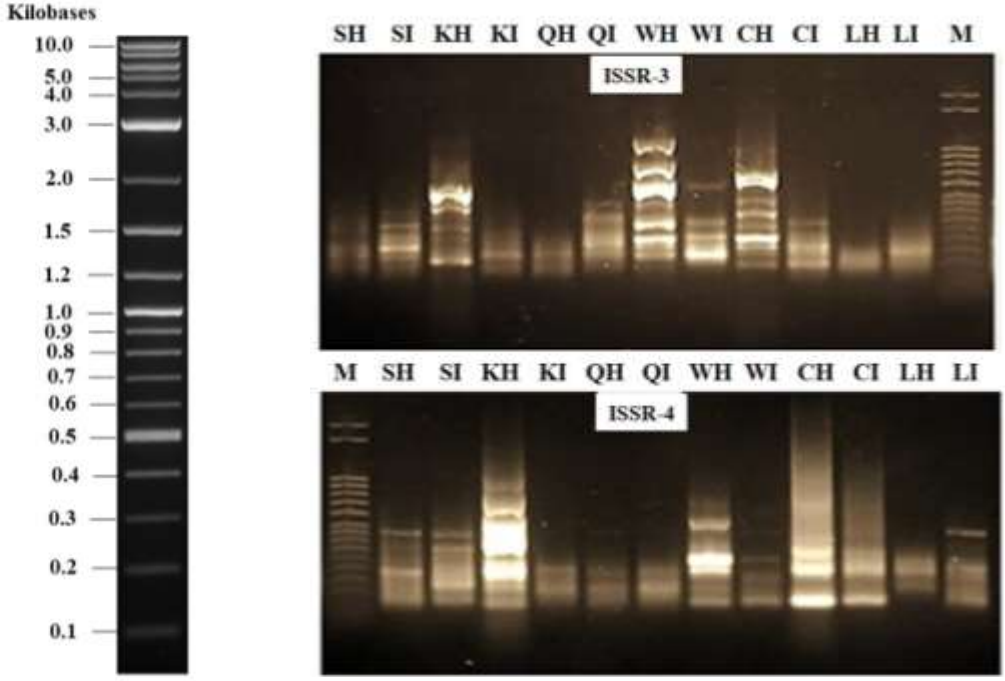

Fig. 3a. DNA polymorphisms of the six cucurbit species infected with *Zucchini yellow mosaic virus* (ZYMV) compared to healthy using five inter simple sequence repeat (ISSR) (3 and 4). Where; SH: Squash healthy; SI: Squash infected; KH: Kantaloupe healthy; KI: Kantaloupe infected; QH: Qethaa healthy; QI: Qethaa infected; WH: Watermelon healthy; WI: Watermelon infected; CH: Cucumber healthy; CI: Cucumber infected; LH: Luffa healthy; LI: Luffa infected, and M, 1 Kb plus DNA Ladder

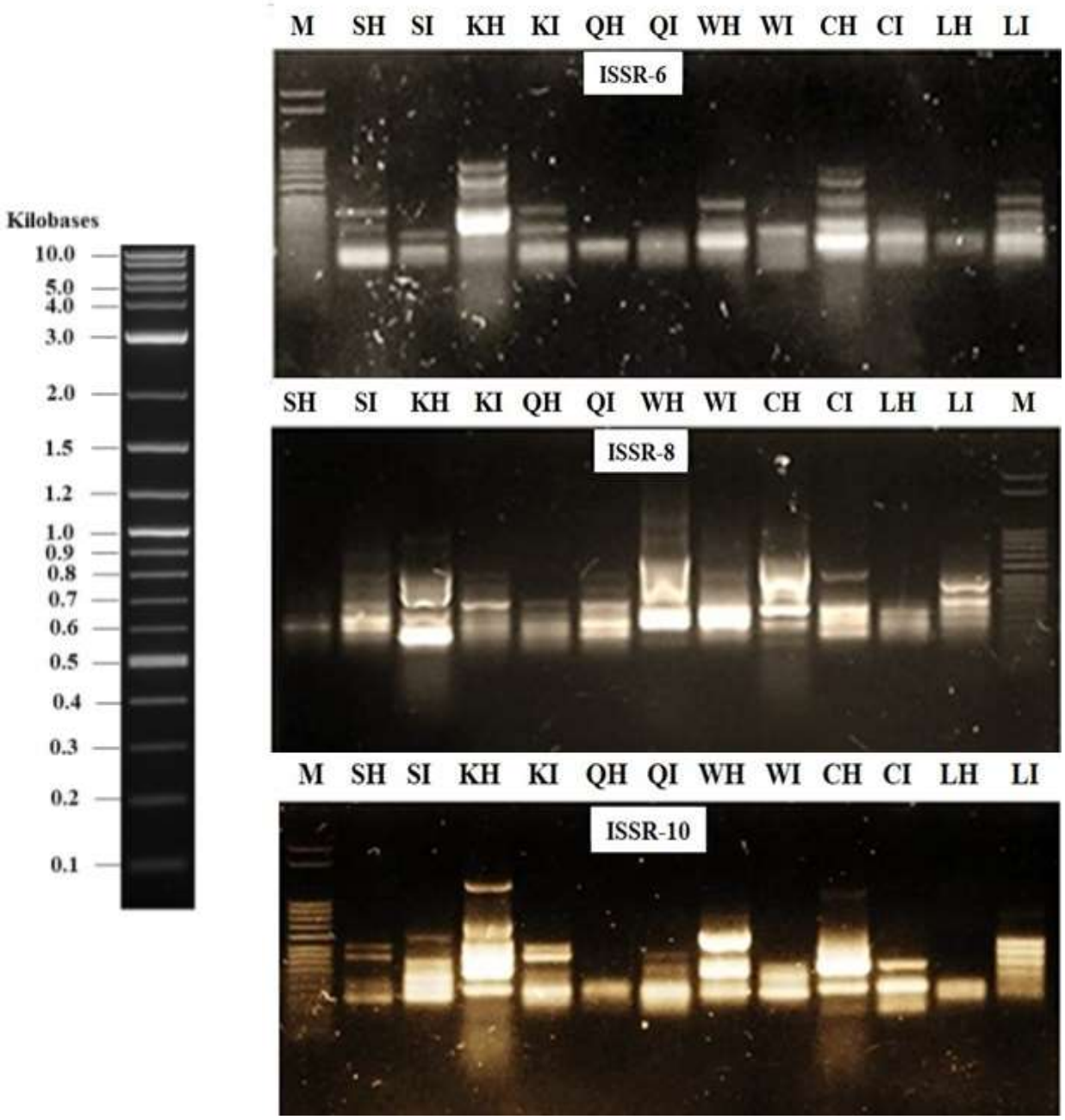

Fig. 3b. DNA polymorphisms of the six cucurbit species infected with *Zucchini yellow mosaic virus* (ZYMV) compared to the healthy ones using five inter simple sequence repeat (ISSR) primers (6, 8 and 10). Where; SH: Squash healthy; SI: Squash infected; KH: Kantaloupe healthy; KI: Kantaloupe infected; QH: Qethaa healthy; QI: Qethaa infected; WH: Watermelon healthy; WI: Watermelon infected; CH: Cucumber healthy; CI: Cucumber infected; LH: Luffa healthy; LI: Luffa infected, and M: 1 Kb plus DNA Ladder

Table 2: Total amplified fragments (TAFs) of DNA polymorphisms of the six tested cucurbit species infected with *Zucchini yellow mosaic virus* (ZYMV) compared to the healthy ones using five inter simple sequence repeat (ISSR) primers

Primers	Plant samples												
	TAFs	SH	SI	KH	KI	QH	QI	WH	WI	CH	CI	LH	LI
ISSR-03	10	1	1	4	6	4	8	3	2	2	4	3	1
ISSR-04	7	3	3	7	1	2	1	2	4	3	1	1	2
ISSR-06	7	2	2	5	3	1	2	1	3	3	1	1	3
ISSR-08	7	3	1	2	4	2	6	5	4	2	4	4	1
ISSR-10	7	4	5	5	3	1	4	4	3	4	3	1	4
Total	38	13	12	23	17	10	21	15	16	14	13	10	11

Where; TAFs: Total amplified fragments; SH: Squash healthy; SI: Squash infected; KH: Kantaloupe healthy; KI: Kantaloupe infected; QH: Qethaa healthy; QI: Qethaa infected; WH: Watermelon healthy; WI: Watermelon infected; CH: Cucumber healthy; CI: Cucumber infected; LH: Luffa healthy. LI: Luffa infected

Table 3: Identities (%) of DNA polymorphisms of six cucurbit species infected with *Zucchini yellow mosaic virus* (ZYMV) compared to the healthy ones using five inter simple sequence repeat (ISSR) primers

Plant samples	Plant samples											
	SH	SI	KH	KI	QH	QI	WH	WI	CH	CI	LH	LI
SH	100											
SI	56	100										
KH	61	51	100									
KI	53	41	45	100								
QH	45	29	50	46	100							
QI	47	48	41	74	40	100						
WH	64	50	57	55	29	48	100					
WI	71	67	63	56	50	56	59	100				
CH	52	62	65	45	26	40	77	55	100			
CI	46	40	33	67	36	71	40	43	30	100		
LH	43	18	42	44	53	45	36	56	33	26	100	
LI	75	61	65	43	40	44	70	62	56	33	29	100

Where; SH: Squash healthy; SI: Squash infected; KH: Kantaloupe healthy; KI: Kantaloupe infected; QH: Qethaa healthy; QI: Qethaa infected; WH: Watermelon healthy; WI: Watermelon infected; CH: Cucumber healthy; CI: Cucumber infected; LH: Luffa healthy. LI: Luffa infected

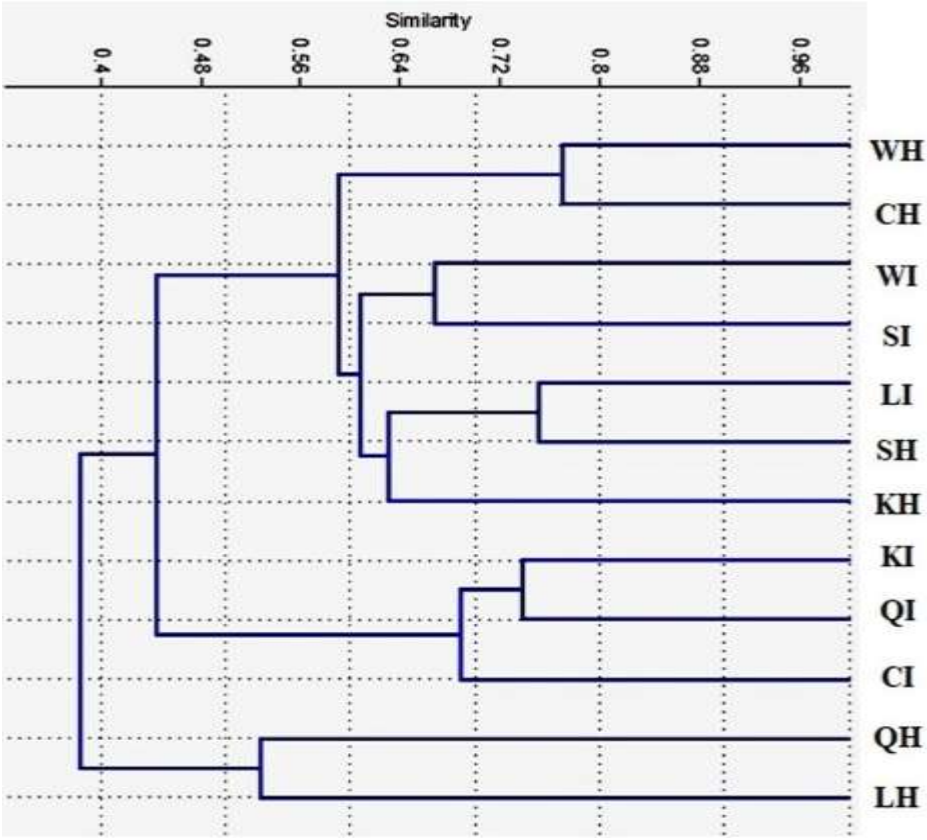

Fig. 4. A phylogenetic tree of DNA polymorphisms of the six cucurbit species infected with *Zucchini yellow mosaic virus* (ZYMV) compared to the healthy ones using five ISSR primers. Where; SH: Squash healthy; SI: Squash infected; KH: Kantaloupe healthy; KI: Kantaloupe infected; QH: Qethaa healthy; QI: Qethaa infected; WH: Watermelon healthy; WI: Watermelon infected; CH: Cucumber healthy; CI: Cucumber infected; LH: Luffa healthy; LI: Luffa infected

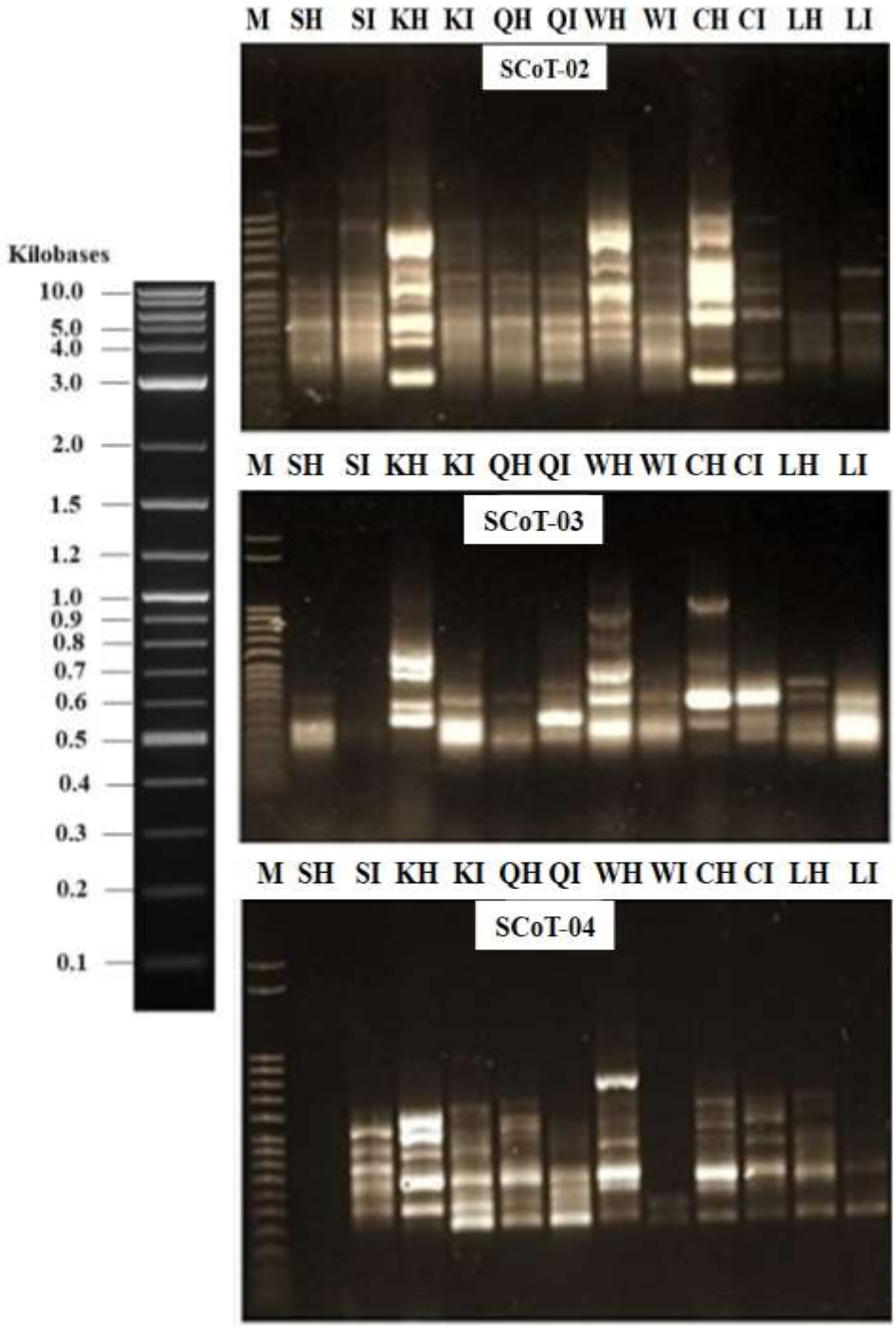

Fig. 5a. DNA polymorphisms of the six tested cucurbit species infected with *Zucchini yellow mosaic virus* (ZYMV) compared to the healthy ones using three SCoT primers (2, 3 and 4). M, 1 Kb Plus DNA Ladder. Where; SH: Squash healthy; SI: Squash infected; KH: Kantaloupe healthy; KI: Kantaloupe infected; QH: Qethaa healthy; QI: Qethaa infected; WH: Watermelon healthy; WI: Watermelon infected; CH: Cucumber healthy; CI: Cucumber infected; LH: Luffa healthy; LI: Luffa infected.

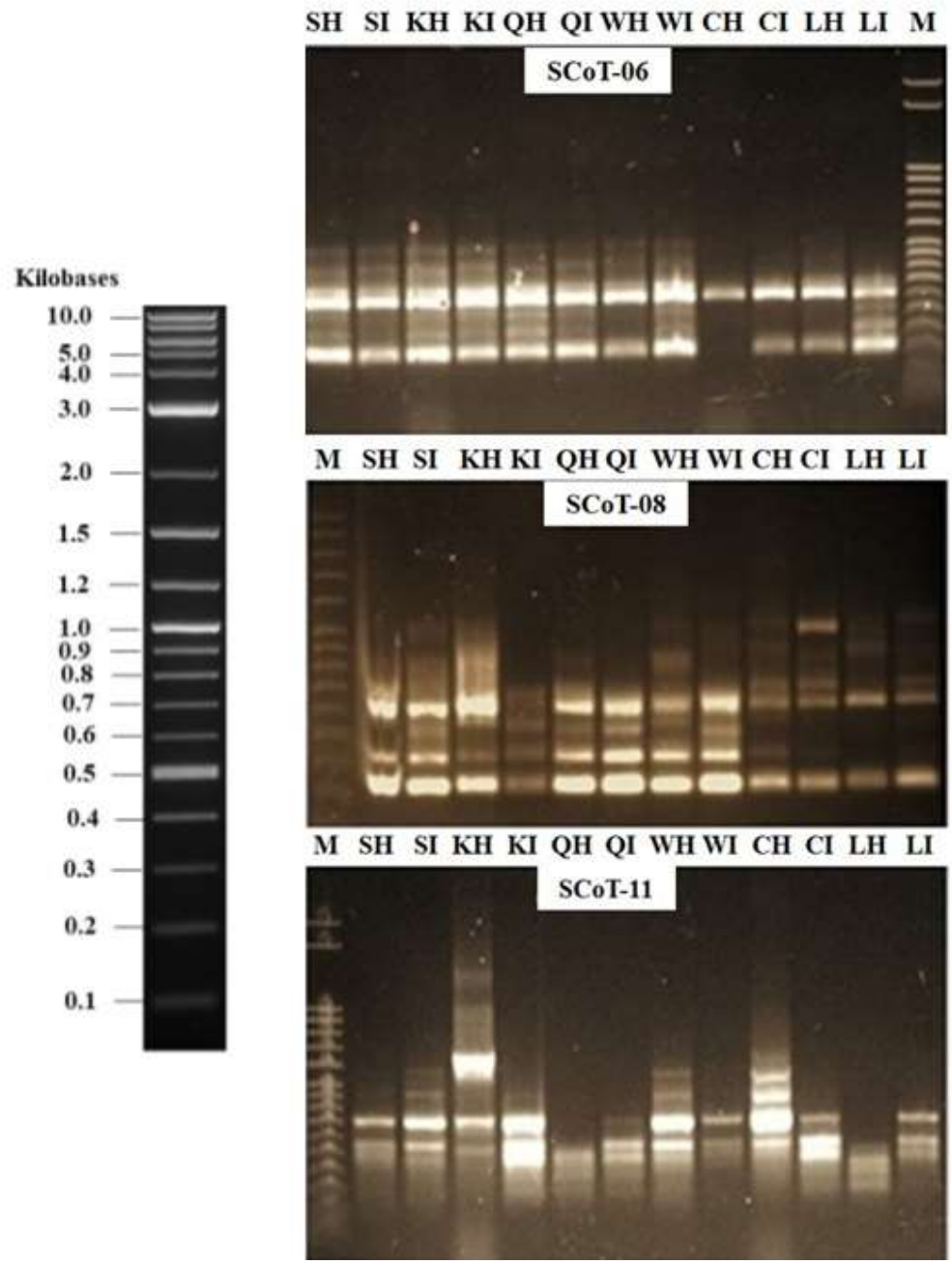

Fig. 5b. DNA polymorphisms of the six tested cucurbit species infected with *Zucchini yellow mosaic virus* (ZYMV) compared to the healthy ones using three SCoT primers (6, 8 and 11). M, 1 Kb Plus DNA Ladder. Where; SH: Squash healthy; SI: Squash infected; KH: Kantaloupe healthy; KI: Kantaloupe infected; QH: Qethaa healthy; QI: Qethaa infected; WH: Watermelon healthy; WI: Watermelon infected; CH: Cucumber healthy; CI: Cucumber infected; LH: Luffa healthy; LI: Luffa infected

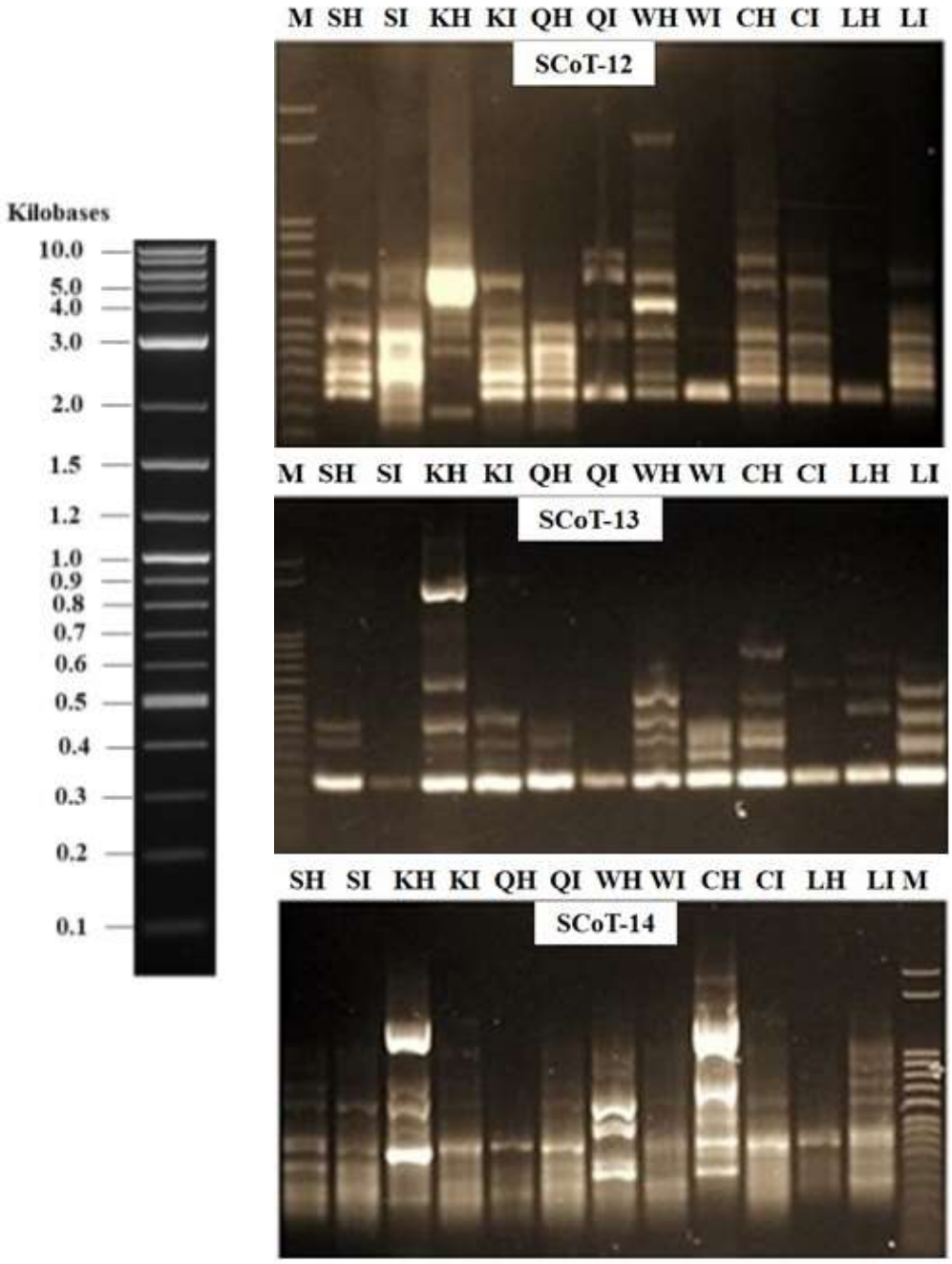

Fig. 5c. DNA polymorphisms of the six tested cucurbit species infected with *Zucchini yellow mosaic virus* (ZYMV) compared to the healthy ones using three SCoT primers (12, 13 and 14). M, 1 Kb Plus DNA Ladder. Where; SH: Squash healthy; SI: Squash infected; KH: Kantaloupe healthy; KI: Kantaloupe infected; QH: Qethaa healthy; QI: Qethaa infected; WH: Watermelon healthy; WI: Watermelon infected; CH: Cucumber healthy; CI: Cucumber infected; LH: Luffa healthy; LI: Luffa infected

Table 4: Total amplified fragments (TAFs) of DNA polymorphisms of six cucurbit species infected with *Zucchini yellow mosaic virus* (ZYMV) compared to the healthy ones using nine start codon targeted (SCoT) primers

Primers	TAF	Plant samples											
		SH	**SI**	**KH**	**KI**	**QH**	**QI**	**WH**	**WI**	**CH**	**CI**	**LH**	**LI**
SCoT-02	13	6	7	10	6	6	8	7	5	8	7	2	4
SCoT-03	07	2	1	5	3	2	3	4	2	4	3	3	3
SCoT-04	10	0	5	4	5	5	4	6	2	5	6	4	2
SCoT-06	06	4	4	2	5	4	4	4	6	6	6	4	6
SCoT-08	09	3	5	4	4	4	4	3	4	5	5	3	5
SCoT-11	10	3	5	6	6	2	3	5	2	4	5	3	3
SCoT-12	15	6	8	7	7	6	7	11	2	6	8	1	7
SCoT-13	08	3	5	6	6	2	3	5	2	4	5	3	3
SCoT-14	10	5	4	5	2	4	3	4	4	9	2	1	7
Total	**88**	**32**	**44**	**49**	**44**	**35**	**39**	**49**	**29**	**51**	**47**	**24**	**40**

Where; SH: Squash healthy; SI: Squash infected; KH: Kantaloupe healthy; KI: kantaloupe infected; QH: Qethaa healthy; QI: Qethaa infected; WH: Watermelon healthy; WI: Watermelon infected; CH: Cucumber healthy; CI: Cucumber infected; LH: Luffa healthy; LI: Luffa infected

Table 5: Identities (%) of DNA polymorphisms of the six tested cucurbit species infected with *Zucchini yellow mosaic virus* (ZYMV) compared to the healthy ones using nine start codon targeted (SCoT) primers

Plant samples	Identities (%) of DNA polymorphisms											
	SH	SI	KH	KI	QH	QI	WH	WI	CH	CI	LH	LI
SH	100											
SI	64	100										
KH	45	56	100									
KI	51	55	57	100								
QH	63	57	52	67	100							
QI	64	69	63	71	73	100						
WH	60	65	67	59	69	62	100					
WI	72	60	50	57	59	72	60	100				
CH	56	59	70	56	65	66	72	60	100			
CI	64	59	51	70	76	79	62	68	75	100		
LH	49	53	32	45	67	62	54	55	43	67	100	
LI	68	57	41	50	57	69	58	68	62	73	67	100

Where; SH: Squash healthy; SI: Squash infected; KH: Kantaloupe healthy; KI: kantaloupe infected; QH: Qethaa healthy; QI: Qethaa infected; WH: Watermelon healthy; WI: Watermelon infected; CH: Cucumber healthy; CI: Cucumber infected; LH: Luffa healthy; LI: Luffa infected

A total of 88 DNA fragments distributed as follows: 13, 7, 10, 6, 9, 10, 15, 8, and 10 were generated using SCoT-02, SCoT-03, SCoT-04, SCoT-06, SCoT-08, SCoT-11, SCoT-12, SCoT-13, and SCoT-14, respectively. Based on the number of amplified fragments, the samples could be classified into two groups; the 1st group included ZYMV-infected samples of each of squash, kantaloupe, luffa, and qethaa, which appeared in 44, 44, 40 and 39 fragments that were higher than the healthy samples of the same species, and generated 32, 49, 24, and 35 fragments, respectively. The 2nd group included the healthy samples of each of cucumber, cantaloupe, and watermelon that recorded 51, 49, and 49 fragments, compared to the ZYMV-infected samples, which showed 47, 44, and 29 DNA fragments. A 100 % was recorded for the polymorphic DNA fragments, because no monomorphic fragments were recorded. The lowest healthy % of the polymorphic fragments (27.27 %) was recorded for the healthy luffa, followed by squash (36.36 %), and qethaa (39.77 %).

Using SCoT assessment, the DNA polymorphisms of the six cucurbit species infected with ZYMV showed identities that ranged from 32 to 79 % compared to the healthy species using nine SCoT primers. The lowest % was recorded between the healthy kantaloupe (32 %) and cucumber (43 %), while the highest % was recorded between ZYMV-infected qethaa (79 %) and cucumber (73 %). As overall, the identities % recorded among the tested cucurbit samples when assessed using SCoT were higher than those determined when assessed using ISSR-PCR. This was also obvious from the average of DNA fragments generated via ISSR technique (7.6) compared to SCoT (14).

When the ZYMV-infected plant samples of each cucurbit species were compared to the healthy cucurbits of the same species, identities of 75 followed by 73, 67, 64, 60, and 57 % were observed among the samples of cucumber, qethaa, luffa, squash watermelon, and kantaloupe, respectively.

Results demonstrated in Fig. (6) represent the phylogenetic tree of DNA polymorphisms of the six cucurbit species infected with ZYMV compared to the healthy ones using nine SCoT primers. It was noted that the members of clusters or subclusters obtained via SCoT marker were different from those obtained via ISSR-PCR markers. This may attributed to the location the healthy and ZYMV-infected samples together in the same sub-cluster, and thus there is a need to use a large number of ISSR-PCR primers with different oligonucleotides, which differ from the currently applied primers.

4. Discussion

Several investigations have used the biochemical protein markers to assess the genetic variability of the different plants using SDS-PAGE (Shaban *et al*., 2022; Wang *et al*., 2023; El-Masry *et al*., 2024). This technique has been recorded as a quick and accurate method based on the protein profiles; in addition, it has no any environmental effect, and is characterized by its validity and simplicity for distinguishing among the genetic structure of the plants (Zahoor *et al*., 2022; Iqbal *et al*., 2024; Ihsan *et al*., 2024).

In this study, SDS-PAGE analysis was found to be suitable for determining the genetic variations among ZYMV-infected qethaa, luffa, squash, and kantaloupe plants, compared to the healthy plants. Results showed that a total of 15 scorable protein patterns were recorded with averages of 8 and 9.25 bands for the healthy and ZYMV-infected cucurbit species, respectively. The protein patterns of the healthy species of the tested cucurbits were higher than the infected species. These observations were confirmed upon comparison of percentages of similarities of ZYMV-infected (43-71 %) cucurbit species compared to the healthy ones (59-83 %). This was also documented by results of phylogenetic trees of the protein profiles of the four ZYMV-infected cucurbit plants and those of the healthy species gathered into the same subcluster.

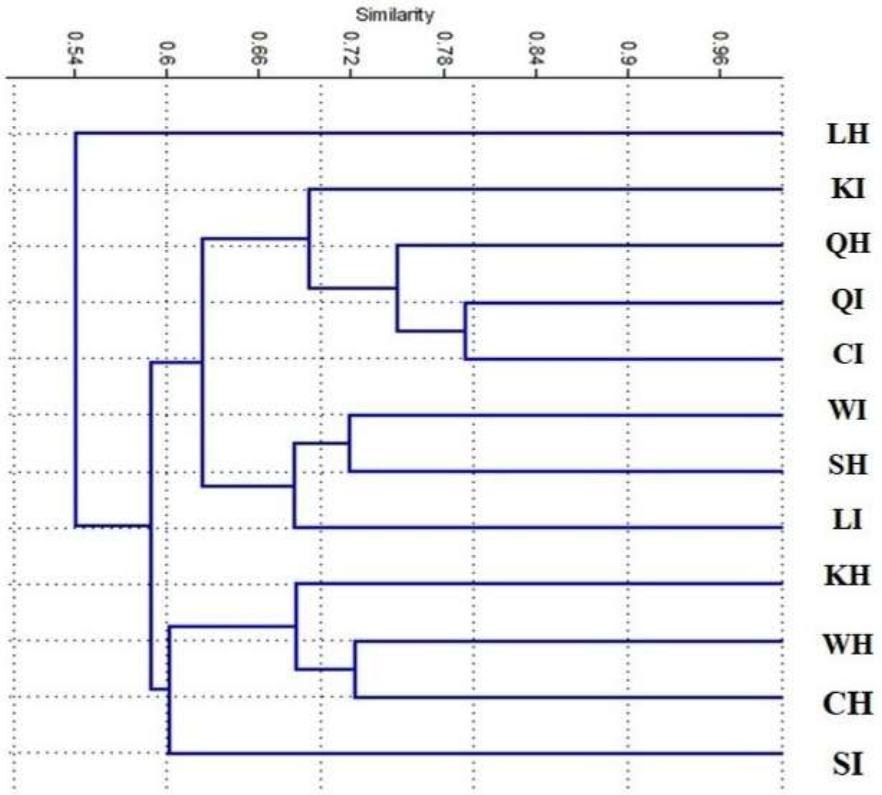

Fig. 6. A phylogenetic tree of DNA polymorphisms of the six cucurbit species infected with *Zucchini yellow mosaic virus* (ZYMV) compared to the healthy species using nine SCoT primers. Where; SH: Squash healthy; SI: Squash infected; KH: Kantaloupe healthy; KI: Kantaloupe infected; QH: Qethaa healthy; QI: Qethaa infected; WH: Watermelon healthy; WI: Watermelon infected; CH: Cucumber healthy; CI: Cucumber infected; LH: Luffa healthy; LI: Luffa infected

Based on these findings, SDA-PAGE analysis was proved to be suitable for differentiation between the different cucurbits whatever healthy or ZYMV-infected. These results are in harmony with those of Vinayashree and Vasu, (2021). The experimental results of this study could be supported by those of El-Masry *et al*., (2024), who used the SDS-PAGE analysis to differentiate among the banana bunchy top virus-infected bananas compared to the healthy ones. According to the previous studies, it was observed that the methods based on DNA, *i.e*., molecular markers, were used to determine the genetic diversity of the cucurbit plants, compared to the morphological characteristics (Zhu *et al*., 2021; Castellanos-Morales *et al*., 2024). Of these molecular marker methods, inter simple sequence repeat (ISSR) (Venkatesan *et al*., 2021; Amruthakumar *et al*., 2023), restriction fragment length polymorphisms (RFLP) (Abdulkina *et al*., 2023), simple sequence repeats (SSR) (Raghami *et al*., 2014; Tahir *et al*., 2022), and random amplified polymorphic DNA (RAPD) (Fadel *et al*., 2022) have been used to evaluate the genetic diversity among the different species of *Cucurbitaceae*.

In this study, upon using ISSR marker to differentiate among the six cucurbit species (healthy or ZYMV-infected), about 38 polymorphic DNA fragments were generated using five ISSR primers. No monomorphic DNA fragments were amplified in the presence of DNA extracts of the six cucurbits as templates via ISSR-PCR. The cucurbit watermelon species showed the highest identity % between the ZYMV-infected and the healthy samples, followed by squash, kantaloupe, qethaa, cucumber, and luffa. As an overall view, ISSR-03 primer generated a DNA polymorphism containing 10 DNA fragments while the other four primers (ISSR-04, ISSR-06, ISSR-08, and ISSR-10) showed equal number of DNA fragments within their polymorphisms (7 for each). It is worth mentioning that all DNA fragments were of polymorphic type and no unique DNA markers were recorded. The phylogenetic tree of DNA polymorphisms confirmed the genetic relationship among each of the healthy samples of watermelon and cucumber, the healthy samples of qethaa and cucumber; ZYMV-infected samples of watermelon and squash, and ZYMV-infected samples of kantaloupe and qethaa.

Results of this study agreed with those previously reported by several investigations, which proved that ISSR was successfully applied to determine the genetic diversity or homogeneity of the different plants (Gupta *et al*., 2021; Shaban *et al*., 2022; Kulyan *et al*., 2023). ISSR was also recommended by Mahfouzea *et al*., (2018) for determining the genomic DNA variation in ZYMV-infected plants. The genetic diversity of melon (*Cucumis melo* L.) was determined by Maleki *et al*., (2017) using 23 ISSR primers, in which 10 ISSR primers were observed based on the degree of polymorphism. A previous study reported by Payel *et al*., (2015) had applied ISSR method to determine the genetic relationships among the cucurbit cultivars. Using five ISSR-PCR primers, a total of 117 DNA fragments were recorded, where 57 of them were polymorphic.

It is worth mentioning that ISSR markers have had a greater robustness in repeatability due to their high variability (Bornet and Branchard, 2001). One can recommend ISSR molecular markers as being more benefitable, as they provide valuable site information, genetic polymorphism, and reveal the various microsatellite variations among the individuals (Verma *et al*., 2017). Dje *et al*., (2006) employed ISSR markers to determine the genetic variability of three African edible-seeded cucurbits (*Cucumis melo*, *Citrullus lanatus* L., and *Cucumeropsis mannii* L.) by using 1.5 % agarose gels, 21 ISSR-PCR primers, and electrophoresing at 80 V for 5 h. They obtained 11 ISSR markers at an annealing temperature that varied from 50 to 52 °C. Dje *et al*., (2010) evaluated the genetic diversity of four accessions of edible seeded *Citrullus lanatus* that were originated from Côte d'Ivoire using 20 ISSR-PCR primers. These primers generated 258 DNA fragments including 258 DNA polymorphic bands. The pairwise genetic distance among the evaluated individuals ranged from 0.0 to 0.61.

The SCoT DNA marker is a technique established by Collard and Mackill, (2009) based on short nucleotides around the start codon "ATG" of the conserved regions of the plant genes. These nucleotides have been applied as single primers to generate DNA polymorphisms from the genome regions that are able to differentiate among the plant species (Faiesal and Sadik, 2024). A study conducted by Abd El-Moneim *et al*., (2021) revealed that the start codon had targeted (SCoT) polymorphism marker as an important fingerprinting method upon studying the plant genetics, genomics, and molecular breeding during the last 8-10 years. The same author stated that SCoT marker can distinguish among the genetic variations in a specific gene that links to a specific trait, because it targets the region flanking the start codon in the plant genes. Therefore, SCoT is a novel, simple, cost-effective, highly polymorphic, and reproducible molecular marker, and there is no need for prior sequence information.

In this study and using nine SCoT markers, DNA fingerprinting of ZYMV-infected cucurbits compared to the healthy plants of the same species was applied for determining their genetic variations. Results showed that 88 polymorphic DNA fragments were generated with an average of 9.77 for each primer, and the observed similarities among them ranged from 32 to 79 %.

Currently, when the results of SCoT were compared to those of ISSR, it is observed that the SCoT was more informative than the ISSR marker in evaluating the genetic diversity of the healthy and virus-infected cucurbit samples, which may be attributed to the fact that SCoT showed identities (%) that were higher than those determined using ISSR. These results agree with those of Igwe *et al*., (2017) who conducted a comparative study between SCoT and ISSR markers and revealed that ISSR markers were less efficient than SCoT markers in assessment of the genetic diversity of *Vigna unguiculata*. This was also supported by the results of Gogoi *et al*., (2020), who reported that SCoT markers displayed higher level of discriminatory and informative values than ISSR and RAPD markers.

In Egypt, El-Masry *et al*., (2024) evaluated the genetic diversity of BBTV-infected banana cultivars (Edward Cavendish, Maghrabi, and Williams) compared to the healthy ones using SCoT DNA primers. The results showed that a number of 70 scorable DNA fragments were generated with an average of 14 DNA fragments for each primer. They also noted that the average of DNA amplified fragments of the healthy banana cultivars was higher than that of BBTV-infected cultivars. Based on the obtained findings in this study, SDA-PAGE analysis, ISSR, and SCoT markers were proved to be suitable for differentiation among the different cucurbits whatever they were healthy or ZYMV-infected. Meanwhile, SCoT markers were proved to be more informative than ISSR.

Conclusion

The effect of ZYMV infection on the genome of some cucurbit species was determined using three molecular tools that dependent on protein (SDS-PAGE) and DNA (ISSR and SCoT markers). This was accomplished via determining the genetic variations of ZYMV-infected cucurbit species compared to the healthy plants of the same species. Both of the protein patterns and DNA polymorphisms were generated using five ISSR and nine SCoT primers, which were analyzed for assessment of the DNA fingerprinting of ZYMV-infected and healthy species. SCoT was more informative than ISSR marker for differentiation among the infected and healthy cucurbit species.

Acknowledgments

The authors would like to acknowledge Dr. Gehan Zenhom, Associate Professor of Horticulture, Institute of Horticultural Research, Agricultural Research Center (ARC), Giza, Egypt, for kindly providing us with the cucurbit species used in this study.

Conflict of interests

According to the authors, there are no conflicts of interest.

Funding source

Self-funded.

Ethical approval

None-applicable.

Authors' Contributions

Conceptualization: S.F., S.I., A.S. and M.A.; Investigation: S.F., S.I., A.S, M.A. Supervision: S.I., A.S, M.A.; Writing, reviewing, and editing: S.M., S.A.., S.I. and M.A.; Roles/Writing -original draft: S.F; A.S. and M.A.

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