



Genetic diversity studies and screening for rice stem borer (*Chilo Agamemnon*) resistance in six Egyptian rice cultivars (*Oryza sativa* L.) using DNA based-markers

Reda E. A. Moghaieb · Etr H. K. Khashaba · Amany M. Abd El Azim · Sanaa A. Ibrahim

Received: 24 March 2020 / Accepted: 23 January 2021 / Published online: 15 February 2021
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Abstract In order to assess the genetic diversity among six Egyptian rice cultivars and developing cultivars resistant to different rice stem borers, random amplified polymorphic DNA (RAPD), sequence-related amplified polymorphism (SRAP) and simple sequence repeats (SSR) analyses was resolved. The rice cultivars tested showed 80, 68 and 64 polymorphism percentages amongst them as revealed by rice marker SSR, SRAP and RAPD analyses respectively. The SSR, SRAP and RAPD analyses showed 18, 8 and 17 genotype-specific markers respectively. The dendrogram constructed based on the statistics gathered from different molecular markers explain the genetic relationship amongst the six Egyptian rice cultivars. The rice genome was screened for ten SSR locus associated with insects resistance genes which is located in distinctive chromosomes started from chromosome number 7 to number 12. The cultivars showed different number of alleles and Sakha 103 exhibited the highest allelic number (17 alleles) compared to the other cultivars tested. The genotype specific markers and the insect associated SSR

markers can be used for cultivar identification at the molecular level and can be considered as useful markers for rice breeding and the effective management strategy to control pest.

Keywords Egyptian rice · Genetic diversity · SSR · SRAP · RAPD markers

Introduction

Raising productivity and reducing yield losses due to insect pest and disease attack are necessary for rice breeders to meet the global rice demand. It is estimated that biotic stress alone can cause yield loss of up 52% of the global rice production of which 25% is attributed to the attack of insect pests (Yarasi et al. 2008). Damage caused by stem borers (*Chilo agamemnon*) which is a key pest in Egypt infests rice stem during the vegetative stage, it tends to destroy the stems by boring holes through the leaf sheaths at the nodal region, leading to a destructive effect on the terminal shoots. The damage symptom known as “dead heart” and resulting in white head symptom during productive stage (Chaudhary et al. 1984; Elanchezhyan and Arumugachamy 2015; RRTC 2015).

Genetic diversity of rice genotypes has been studied based on simple sequence repeats (SSR) markers linked to insect’s resistance genes, which can detect

R. E. A. Moghaieb
Department of Genetics, Faculty of Agriculture, Cairo
University, Giza, Egypt

E. H. K. Khashaba (✉) · A. M. Abd El Azim ·
S. A. Ibrahim
Plant Protection Research Institute, Agriculture Research
Center, Giza, Egypt
e-mail: ater_hk@yahoo.com

high allelic variation and provide a tool for gene mapping and marker—assisted selection (MAS) in rice. There is limitation about connecting the breeding program with the molecular data in Egypt (Temnykh et al. 2000; Shabanimofrad et al. 2015). Also, sequence-related amplified polymorphism (SRAP) is used to amplify coding regions of DNA with primers targeting open reading frames. These markers have been confirmed to be robust and highly variable (Yu et al. 2008; Robarts and Wolfe 2014). And random-amplified polymorphic DNA (RAPD) markers that appear to estimate more distantly related individual's with intra or inter genetic distances (Fritsch and Rieseberg 1995).

In the present investigation different molecular markers (RAPD, SRAP and SSR) were employed to resolve the genetic variability among six commercial rice cultivars. The genotype specific markers were detected. Also the genome of the different rice genotypes used was screened with SSR primers associated with insect resistance genes.

Materials and methods

Plant materials

Six rice cultivars (*Oryza sativa* L.) which are commonly used in the Egyptian agriculture, namely Giza 177, Giza 178, Giza 179, Sakha 101, Sakha 102 and Sakha 103 were provided by Field Crop Institute, Agricultural Research Center, Ministry of Agriculture, Egypt (Table 1).

DNA extraction

Total genomic DNA was isolated from leaf samples of greenhouse grown plants using the CTAB (Cetyl Trimethyl Ammonium Bromide) method earlier described in Rogers and Bendich (1985). The quality and quantity of DNA were determined using agarose gel (0.8%) electrophoresis and spectrophotometer $1.0 = 50 \mu\text{g/ml}$ at A_{260} , respectively.

RAPD analysis

PCR reaction was performed in a total volume of 20 μl containing 10 ng DNA, 200 μM dNTPs, 1 μM of ten arbitrary 10-mer primers (Operon Technology, Inc.,

Alameda, CA, USA), 0.5 units of Red Hot *taq* polymerase and 10-X *taq* polymerase buffer (AB gene House, UK). For DNA amplification Biometra thermal cycler (2720) was programmed as follow: 94 °C for 5 min followed by 35 cycles 94 °C for 1 min., 35 °C for 1 min., 72 °C for 1 min. and 72 °C for 5 min. (Table 2). The amplification products were analyzed by electrophoresis in 1.2% agarose gel electrophoresis at 50 V, stained by ethidium bromide and photographed under UV light. The sequence of the tested primers was listed in Table 2.

SRAP analysis

A total volume of 20 μl PCR reaction containing 10 ng DNA, 1 μl of dNTPs mixture (10 mM of each dNTP, 1 μl of each SRAP primers, 0.5 U of *taq* polymerase and 2 μl 10 X PCR buffer.

The PCR program consisted of a 5 min of initial denaturation at 94 °C, then five cycles of three steps: 1 min of denaturation at 94 °C, 1 min of annealing at 35 °C, 1 min of extension at 72 °C, followed by further 35 cycles with annealing temperature being increased to 50 °C, with a final extension step of 5 min at 72 °C (Li and Quiros 2001). The PCR products electrophoresed in a 2% agarose gels at 50 V. The gel after staining with ethidium bromide for 30 min visualized by exposure to UV light. The sequence of the tested primers are provide in Table 2.

SSR analysis

Previously selected SSR rice marker which were linked to the insects resistance genes were used in the PCR reactions in a total volume of 20 μl containing 10 ng DNA, 2 μl 10 X buffer; 10 mM dNTPs; 50 mM MgCl_2 , 10 μM each of forward and reverse primers. The PCR profile started with 95 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min extension at 72 °C for 2 min. A final extension of 72 °C for 7 min was included. The PCR products electrophoresed in a 2.5% agarose gels at 90 V. The gel was stained with ethidium bromide for 20 min and observed on a UV trans-illuminator. The sequences of the tested primers was as follows Table 2.

Table 1 The rice cultivars used and their Parentage, pedigree and main features

Cultivars	Parentages	Pedigree	Main features
Sakha 101	100% Japonica	Giza 176/Milang 49	Growth duration (140 days) High blast, stem borers resistant Amylose content (19%)
Sakha 102	100% Japonica	Giza 177/Gz 4098	Short growth duration (125 days) Amylose content (19%) High blast, stem borers resistant
Sakha 103	100% Japonica	Giza 177/Siweon349	High blast, brown spot and stem borers resistant
Giza 177	87.5% Japonica/12.5% Indica	Giza 171/ Yomji No.1/ Pi.No.4	High blast, stem borers resistant Amylose content (18%)
Giza 178	62.5% Indica/37.5% Japonica	Giza 175/ Milang 49	Growth duration (135 days) High blast resistant, stem borer susceptible Amylose content (17%)
Giza 179	75% Indica/25% Japonica	Gz1368/Gz6296	Amylose content (16%) High blast resistant, stem borer moderate susceptible

Band scoring and cluster analysis

The SSR, RAPD and SRAP gel images were scanned using the Gel Doc 2000 Bio-Rad system and were analyzed with Quantity One Software v 4.0.1 (Bio-Rad Laboratories, Hercules, Co. USA). The systat ver. 7-computer program was used to calculate the pairwise differences matrix and plot the dendrogram among rice cultivars (Yang and Quiros 1993). Cluster analysis was based on similarity matrices obtained with the un-weighted pair-group method (UPGMA) using the arithmetic average to estimate the dendrogram.

Results

Genetic diversity among rice cultivars used

Fifteen SRAP primers combination were selected based on their ability to show PCR amplification and reproducible genetic polymorphism among six rice genotypes. Eighty-five SRAP scorable bands were obtained; out of them 58 markers were polymorphic (68%) (Fig. 1; Table 3). The highest bands number of SRAP markers was produced by the primer combination Me2-em1 (10 bands), followed by Me2-em2, Me2-em5, Me2-em3 that shows 9, 8, 7 bands

respectively, while the lowest marker was scored for Me1-em1 and Me3-em5 (4 bands). Seventeen (29%) out of fifty-eight polymorphic SRAP markers generated were found to be genotype specific. The genotype specific SRAP markers are listed in Table 4. Among the specific SRAP markers the highest number of SRAP specific markers was scored for Giza 178 (6 markers) followed by Giza 179 (5 markers) then Sakha 101 (4 markers) and the lowest was scored for Sakha 102 and Sakha103 one marker for each. Also in order to investigate the genetic differences between the six rice genotypes used, random amplified polymorphic DNA (RAPD) analysis was performed. All primers used resulted in the appearance of PCR products with variable number of bands (Fig. 2). Fifty-seven RAPD scorable bands were detected, 37 bands were polymorphic (64%) and can be considered as useful RAPD markers for the tested rice cultivars. Eight out of the thirty seven polymorphic RAPD markers representing (21%) were found to be genotype-specific (Table 5). The highest number of RAPD bands was detected for primers OPE-C-11 (10 bands) followed by OPE-Q-14 (8 bands) while the lowest was scored for OPE-E-06 (3 bands) and they show polymorphism percentages of 90, 87 and 33, respectively. The genotype specific RAPD markers for the different rice cultivars used are listed in Table 6. Among the specific RAPD markers, the highest number of RAPD specific markers was

Table 2 RAPD, SRAP and SSR primers used their sequences

Primers name		Sequence	
<i>RAPD primers</i>			
OPE-B-10			5'-CTGCTGGGAC-3'
OPE-C-02			5'-GTGAGGCGTC-3'
OPE-C-11			5'-AAAGCTGCGG-3'
OPE-E-04			5'-GTGACATGCC-3'
OPE-E-06			5'-AAGACCCCTC-3'
OPE-G-14			5'-GGATGAGACC-3'
OPE-L-04			5'-GACTGCACAC-3'
OPE-M-15			5'-GACCTACCAC-3'
OPE-P-15			5'-GGAAGCCAAC-3'
OPE-Q-14			5'-GGACGCTTCA-3'
Name	Sequence	Name	Sequence
<i>SRAP primers</i>			
me1	5'TGAGTCCAAACCGGATA3'	em1	5'-GACTGCGTACGAATTAAT-3'
me2	5'TGAGTCCAAACCGGAGC3'	em2	5'-GACTGCGTACGAATTTGC-3'
me3	5'TGAGTCCAAACCGGAAT3'	em3	5'-GACTGCGTACGAATTGAC-3'
		em4	5'-GACTGCGTACGAATTTGA-3'
			5'-GACTGCGTACGAATTAAC-3
Name	F-sequence (5'–3')	R-sequence (5'–3')	
<i>SSR primers</i>			
RM 210	TCACATTCGGTGGCATTG	CGAGGATGGTTGTTCACTTG	
RM 544	TGTGAGCCTGAGCAATAACG	GAAGCGTGTGATATCGCATG	
RM 547	TAGGTTGGCAGACCTTTTCG	GTCAAGATCATTCTCGTAGCG	
RM 149	GCTGACCAACGAACCTAGGCCG	GTTGGAAGCCTTTCCTCGTAACACG	
RM 144	TGCCCTGGCGCAAATTTGATCC	GCTAGAGGAGATCAGATGGTAGTGCATG	
RM 320	CAACGTGATCGAGGATAGATC	GGATTTGCTTACCACAGCTC	
RM 167	GATCCAGCGTGAGGAA CACGT	AGTCCGACCACAAGGTGCGTTGTC	
RM 125	ATCAGCAGCCATGGCAGCGACC	AGGGGATCATGTGCCGAAGGCC	
RM 566	ACCCAACACTACGATCAGCTCG	CTCCAGGAACACGCTCTTTC	
RM 261	CTACTTCTCCCTTGTGTGTCG	TGTACCATCGCCAAATCTCC	

scored for Sakha 101, Sakha 102 and Sakha 103 (2 markers) while Giza 178 and Giza 179 scored 1 marker for each one (Table 6).

Screening for insect resistance genes in rice genome

In the present investigation, 10 RM-SSR primers linked to insect's resistant genes from chromosome numbers 7–12 were used to characterize and identify the resistant rice varieties among six rice cultivars.

Twenty one out of 26 alleles were polymorphic (80%). All primers showed different levels of polymorphism except RM 167, RM 149, RM 261 that showed no polymorphism among the six rice cultivars (Table 7; Fig. 3). The data revealed that the primers RM 125 and RM 210 have five alleles compared to other primers that recorded a few allelic numbers. The 10 SSR primer sets which are distributed from chromosome numbers 7–12 revealed 26 alleles and most of the alleles were polymorphic, thus revealing 80% polymorphism. Table 7 shows the distribution of

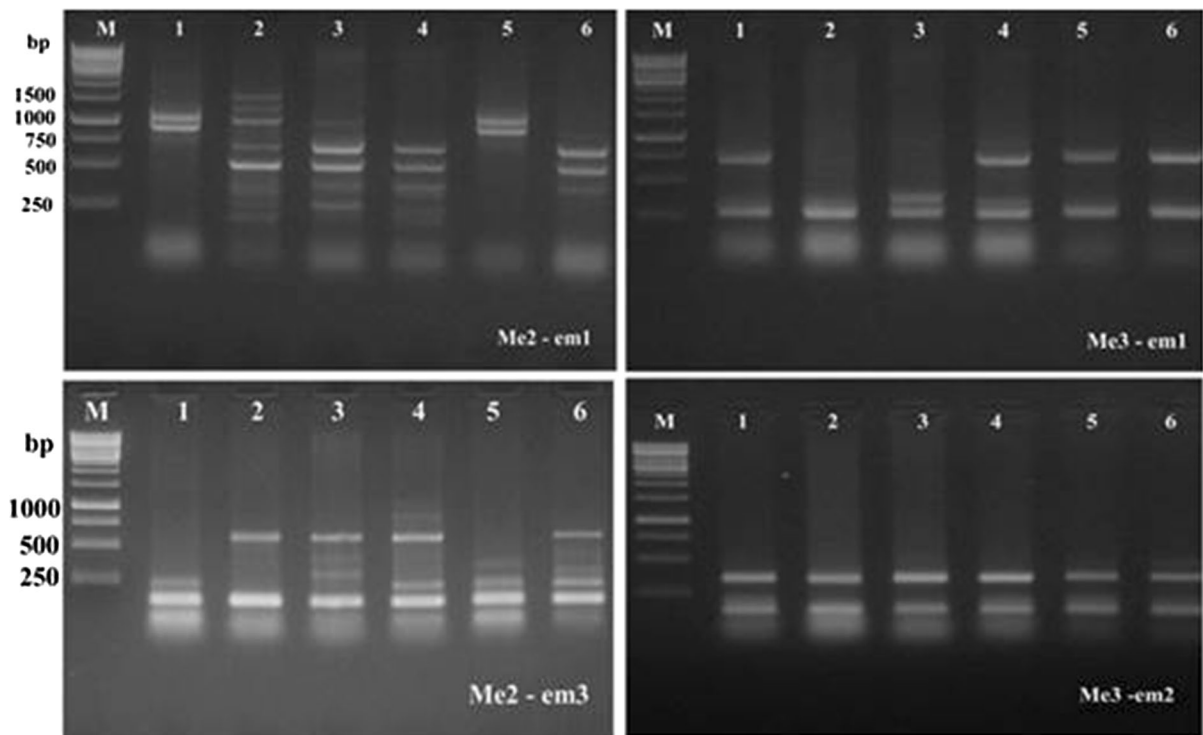


Fig. 1 Genetic polymorphism among rice cultivars as revealed by SRAP analysis. M: 100 bp DNA ladder, 1–6: the rice cultivars Giza-177, Giza-178, Giza-179, Sakha-101, Sakha-102, and Sakha-103 respectively

Table 3 Total number of scorable bands, polymorphism percentage and band size of SRAP markers obtained by fifteen primers

Primers combinations	Total scorable bands	Polymorphic bands	Polymorphism (%)	Band size range
Me1-em1	4	3	75	200–1000
Me1-em2	5	3	60	100–600
Me1-em3	5	3	60	100–700
Me1-em4	5	2	20	200–800
Me1-em5	6	1	33	200–800
Me2-em1	10	10	100	300–800
Me2-em2	9	5	55	250–1000
Me2-em3	7	6	85	200–1010
Me2-em4	5	2	40	300–1000
Me2-em5	8	8	100	500–1250
Me3-em1	4	3	75	250–1100
Me3-em2	3	1	33	300–750
Me3-em3	5	4	85	250–1500
Me3-em4	4	4	100	200–1500
Me3-em5	5	3	60	200–1400
Total	85	58	68	

Table 4 The specific SRAP markers for the six rice genotypes

Genotypes	Markers	Total marker
Giza 177	–	–
Giza 178	Me2em1 (1400 & 1500), Me2,em5 (200, 550, 600 & 700)	6
Giza 179	Me2,em2 (230), me2,em5 (250), Me3,em1 (270), me3,em3 (580) & me3,em4 (250)	5
Sakha 101	Me2,em3 (780 & 1000), me3, em1 (260), me3,em3 (800)	4
Sakha 102	Me2,em2 (510)	1
Sakha 103	Me3,em4 (300)	1
Total		17

polymorphism of SSR markers between different rice chromosomes. Chromosome 7 and chromosome 8 appear to be more polymorphic and chromosome 12 appeared to be the least polymorphism according to the 10 SSR markers used in this study. The genotype-specific SSR markers are listed in Table 8; 18 out of the 28 polymorphic SSR markers generated found to be genotype-specific (64%). The highest number of SSR specific markers was scored for Sakha 103 (8 markers), followed by Giza 178 five markers, Giza 179 three markers, on the other hand, Sakha 101 scored one marker (Table 8). The allelic numbers for the cultivar Sakha 103 was 17 for all the primers and the highest allele counted for primer RM 210 was 4 alleles, while the cultivar Giza178 show the lowest alleles number (15 alleles) (Table 9). Genetic diversity of rice genotypes has been studied based on SSR markers linked to insect's resistance genes and SRAP and RAPD technique. The tree showed a close relationship between resistance (R) and moderately resistant (MR) and susceptible genotypes (S) Fig. 4.

Discussion

Molecular markers have proven to be powerful tools in the assessment of genetic diversity and in elucidation of genetic relationships within and among species (Matin et al. 2012). In the present investigation, SSR, SRAP and RAPD markers were employed to assess the genetic diversity among 6 rice genotypes. And its usage helped in grouping the genotypes according to their subspecies level.

SSR markers are elegant markers for genotype identification and genetic diversity analyses in self-pollinated species (Domini et al. 2000). The procedure

of SRAP primer design is relatively easy and fast since there is no need of prior sequence information and could be used to exhibit the polymorphism degree of the genome more comprehensively due to its capacity in revealing relatively more informative bands no matter on DNA or cDNA level. These markers have proven to be robust and highly variable, and are attained through a significantly less technically demanding process (Li and Quiros 2001; Que et al. 2012; Robarts and Wolfe 2014). The genome of six rice cultivars was analyzed with 15-SRAP primer's combination which resulted in eighty-five SRAP markers, out of them 58 were polymorphic (68%). These results agreed with Fufa et al. (2005) who indicated that SRAP markers have potential for genetic diversity and genotype identification among hard red winter wheat cultivars. Also Zaefizadeh and Goliev (2009) used SRAP to determine the diversity and relationships among 40 sub convers of *Triticum durum* landraces and the polymorphism was 56.7%. The finding of the present investigation agreed with Khan et al. (2016) who found a significant variation among chickpea genotypes using SRAP and AFLP markers which are quite useful for the use in chickpea improving programs. Moreover analysis of the rice genotype used by using RAPD techniques resulted in fifty seven markers among them 37 markers were polymorphic (64%). This agrees with the results obtained by Karande et al. (2017) who assessed the genetic divergence among 12 Indian varieties of rice (*Oryza sativa* L.) using RAPD markers and reported a total of 136 clear score able bands that shows 87.25% polymorphism. Similarly the results of the present work agree with Roopa and Chikkaswamy (2016) as they analyzed the genetic diversity and phylogenetic relationships of 26 selected cultivars of rice (*Oryza*

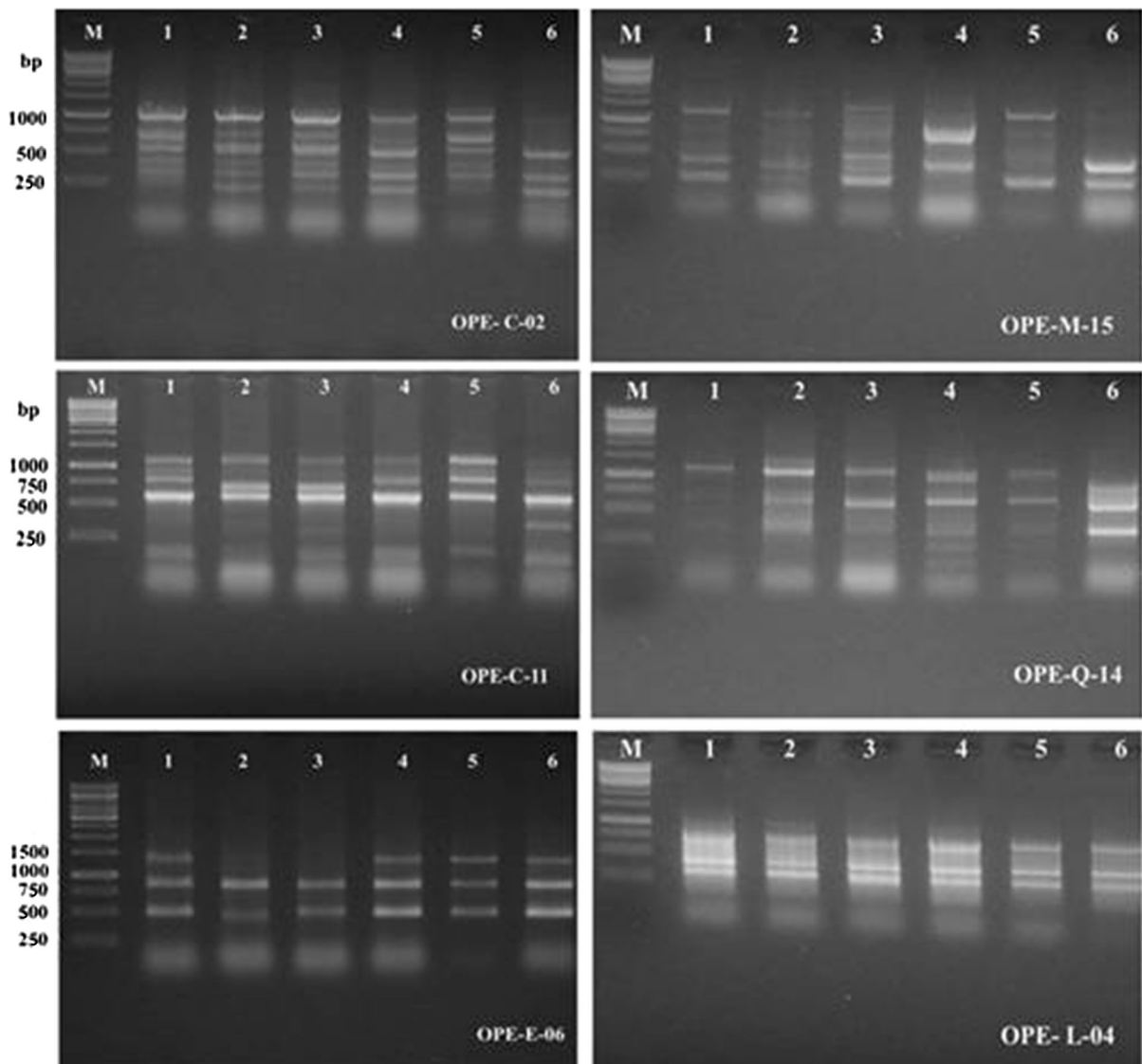


Fig. 2 Genetic polymorphism among rice cultivars as revealed by RAPD analysis. M: 1kbp DNA ladder, 1–6: the rice cultivars Giza-177, Giza-178, Giza-179, Sakha-101, Sakha-102, and Sakha-103 respectively

sativa) by RAPD and found that, 92.2% polymorphism among them. Also Pervaiz et al. (2010) evaluated the genetic polymorphism of 75 rice accessions and improved cultivars using (RAPD) technique which generated a total of 145 RAPD fragments, of which 116 (80%) were polymorphic. Rice stem borers are considered one of the most serious insect pests that attack, damaging the crop from the seedling stage to the maturity. The usage of PCR-based markers for resistance genes allows the MAS for insect resistance in rice to be successfully applied and provide useful

information about the different cultivars that can be used in the breeding programs. The genome of the six rice cultivar used was screened with 10 SSR primers designed specific to stem borers resistant genes on rice genome. The SSR primers used are distributed on different chromosomes from chromosome 7–12. The results indicated that a total of 26 alleles were detected from the six varieties using markers that can be considered as a useful markers for rice insect resistance breeding. The tested rice cultivars differ for the allelic frequencies. The cultivar Sakha 103 which is known to

Table 5 Primers used in RAPD analysis and their number of bands

Primer	Total scorable band	Polymorphic band	Polymorphism %	Band size range
OPE-B-10	4	1	25	300–800
OPE-C-02	7	3	42	250–1000
OPE-C-11	10	9	90	200–1010
OPE-E-04	4	1	25	300–1000
OPE-E-06	3	1	33	500–1250
OPE-G-14	6	5	83	250–1100
OPE-L-04	4	1	25	300–750
OPE-M-15	7	7	100	250–1500
OPE-P-15	5	3	60	200–1500
OPE-Q-14	8	7	87	200–1400
Total	57	37	64	

Table 6 The specific RAPD markers used for the six rice genotypes

Genotypes	Markers	Total marker
Giza 177	–	–
Giza 178	OPE-Q-14 (1400)	1
Giza 179	OPE-C-11 (260)	1
Sakha 101	OPE-G-14 (250), OPQ-14 (200)	2
Sakha 102	OPE-C-11 (240, 600)	2
Sakha 103	OPE-P-15 (200), OPQ-14 (750)	2
Total		8

be insect resistant show the highest number of alleles (17) while the insect sensitive cultivar 178 show the lowest alleles no.15 which matches to the data reported by the Rice Research and Training Center (RRTC), Agricultural Research Center, Egypt (2015)

about to the resistance of the cultivars to insects attack. These variations in multiple allelic levels indicate that SSR marker is crucial for identification of rice accessions at molecular level. The size of the detected alleles across the SSR primers ranged from 100 to 1000 bp, which reflects a large difference in the number of repeats between the different alleles. The genotype specific RAPD, SRAP and SSR markers were determined and these markers can be used for improving rice insect resistance character. Sherif et al. (1999) conclude that in Egypt, Japonica varieties selected from Japanese material were less infested by rice stem borer *Chilo agamemnone* that attack the *indica* varieties. Siwach et al. (2004) who determined the allelic diversity among Basmati and non-Basmati long-grain indica rice varieties using microsatellite markers results were observed in fingerprinting and

Table 7 Total number of alleles detected, polymorphism percentage and molecular size of SSR markers obtained by ten primers

Primer	Chrom. no	Total scorable bands	Polymorphic bands	Polymorphism (%)	Band size range
RM 167	11	1	0	0	190
RM 320	7	2	2	100	190–200
RM 547	8	4	4	100	200–310
RM 144	11	2	2	100	240–260
RM 149	8	1	0	0	270
RM 210	8	5	5	100	120–1000
RM 125	7	5	5	100	110–600
RM 261	12	1	0	0	120
RM 544	8	3	1	33	100–500
RM 566	9	2	2	100	150–260
Total		26	21	80%	

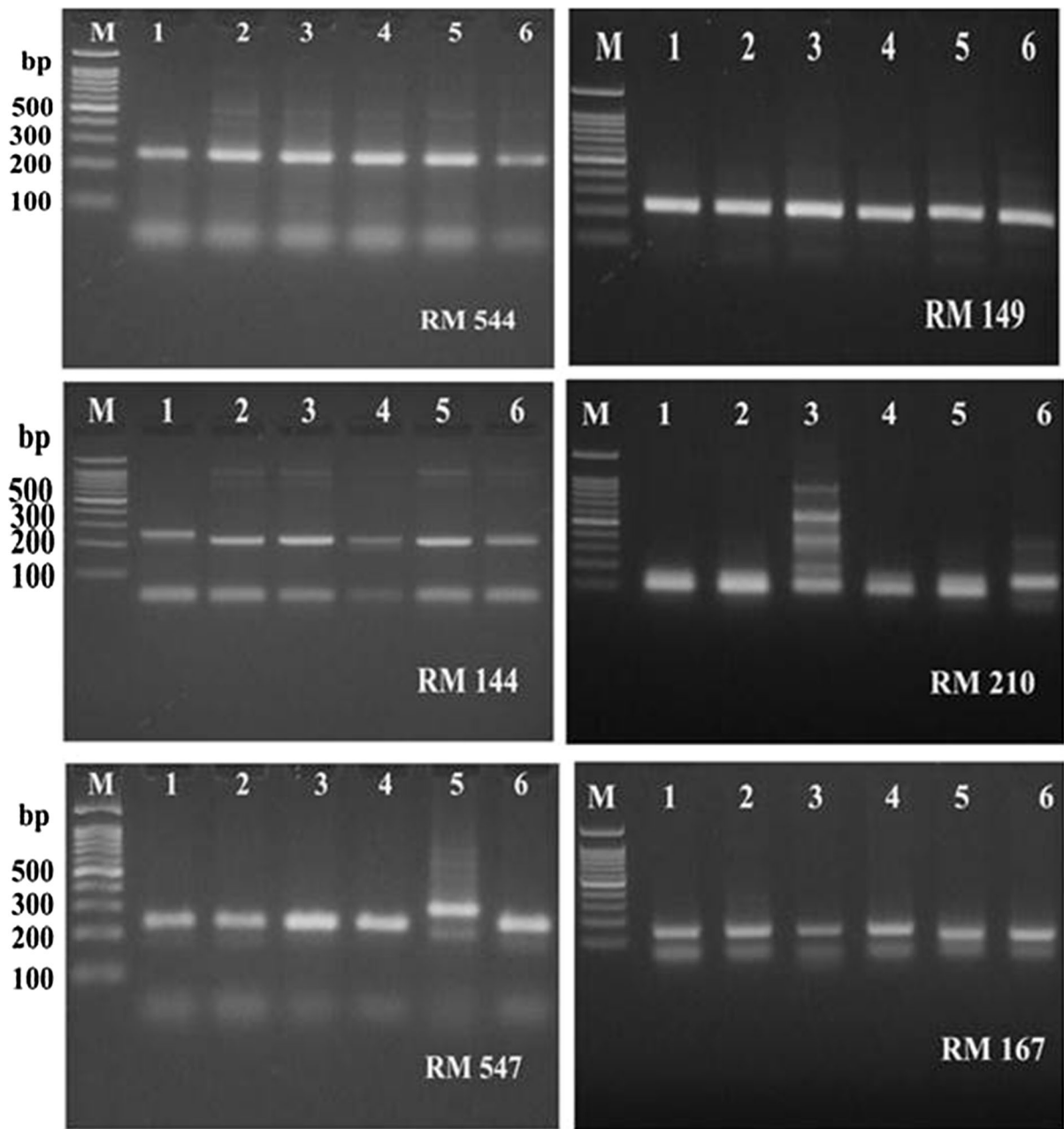


Fig. 3 SSR banding patterns among rice cultivars, M: 100 bp DNA ladder, 1–6: rice cultivars, Sakha-101, Sakha-102, Sakha-103, Giza-177, Giza-178, Giza-179 respectively

diversity studies having 1–8 alleles with an average of 4.58 alleles for various classes of microsatellite, and also 3–9 alleles, with an average of 4.53 alleles per locus for 30 microsatellite markers. Also Kammar and Nitin (2019), studied the polygenic nature of the yellow stem borer (YSB) resistance trait. According to their data, the complex nature of the trait and the

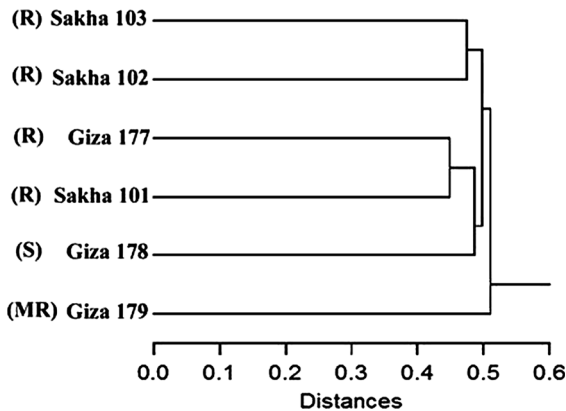
inherent difficulties in screening has consequently made breeding for YSB resistance a difficult task. So they attempt to identify molecular markers linked to major locus conferring YSB resistance in rice using QTL mapping and SCAR markers. From The cluster analysis based on UPGMA dendrogram using the SSR, SRAP and RAPD markers, the six genotypes

Table 8 Rice genotypes and their specific SSR markers

Genotypes	Marker	Total number
Sakha 101	RM144 (240, 260)	2
Sakha102	–	–
Sakha 103	RM210 (160, 200, 1000)–RM566 (150) RM125 (200, 210, 400, 600)	8
Giza 177	–	–
Giza 178	RM320 (190, 200) RM547 (210, 270, 310)	5
Giza 179	RM547 (200)– RM210 (120)– RM544 (100)	3
Total		18

Table 9 Allelic number for each primer per cultivars

Primer name	Sakha 101	Sakha102	Sakha 103	Giza 177	Giza 178	Giza179
RM 167	1	1	1	1	1	1
RM 320	1	1	1	1	1	1
RM 547	1	1	1	1	2	2
RM 144	1	1	1	1	1	1
RM 149	1	1	1	1	1	1
RM 210	2	2	4	2	2	2
RM 125	4	4	2	4	3	4
RM 544	3	3	3	3	2	2
RM 261	1	1	1	1	1	1
RM 566	1	1	2	1	1	1
Total	16	16	17	16	15	16

**Fig. 4** Dendrogram for six rice cultivars constructed from SSR, SRAP and RAPD data analyses using un-weighted pair group arithmetic average similarity matrix computed according to Dice coefficients, showing the relationship between resistance (R) and moderately resistant (MR) and susceptible genotypes (S) rice variety

could be easily categorized into three clusters. Moving closer in terms of similarity, sub-divisions of such

groups can reveal sub-sub groups. Cluster 1 and 2 included genotypes showing resistance, cluster 3 is comprised of one sub clusters included the susceptible and finally the cluster 4 included moderately resistant genotype, Chakravarthi and Naravaneni (2006) revealed that the genotypes that are derivatives of genetically similar type clustered more together using molecular markers.

Conclusion

The fingerprinting results of our study made the characterization of genotypes through DNA markers very easy for estimating the genetic variability and the genetic relationships among six rice cultivars. In breeding insect resistant rice genotypes, the detection of molecular markers linked to genes responsible for resistance is important and affirms with RRTC data on resistance and susceptibility to insects. In addition, in

background selections and crop improvement breeding programs, the Marker Aided Selection (MAS) of plants based on plant genetics is desirable.

Acknowledgements The authors express deep sense of gratitude to Prof. Dr. Abd El Salam E. Draz, Rice Research and Training Center, Agricultural Research Center, Ministry of Agriculture–Egypt, for providing the rice seeds used in this study and constant encouragements. Also Prof. Dr. Ahmed M. El-Sharkawy (Genetic Engineering Research Center, Faculty of Agriculture, Cairo University, Egypt) for helping in critical reading of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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