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Research paper

RAPD Based Molecular Characterization of Three Species of Euphorbia in River Nile State, Sudan

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Abstract

Having a higher discriminating power, molecular techniques such as random amplified polymorphic DNA (RAPD) have been used worldwide as simple and easy to conduct DNA markers to solve problems of differentiating plant materials. Medicinal plants used for centuries to maintain health and to treat diseases, among them Euphorbia family is well known. This study attempted to identify molecular features of selected species from the genus Euphorbia. Three species collected from three sites of River Nile State (*Euphorbia hirta, Euphorbia prostrata and Euphorbia milii*). RAPD-PCR analyses based on 45 primers gave results in term of amplification and polymorphism for the studied plant materials.

Keywords: Euphorbia; RAPD; Polymorphism

توصيف جزيئي مبني على واسمات التضاعف العشوائي متعدد التشكل (RAPD) لبعض انواع نبات ام لبينة (Euphorbia) بولاية نهر النيل، السودان

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المستخلص

لمقدرتها العالية على التمييز تعتبر التقنيات الجزيئية كواسمات التضاعف العشوائي متعدد التشكل من التقنيات واسعة الاستخدام عالميا لبساطتها وسهولة اجرائها للتفريق بين المجموعات النباتية. النباتات ذات الاستخدام الطبي ظلت لقرون تستخدم للعلاج وتصحيح الابدان، من هذه المجموعة تعتبر نباتات ام لبينة (ايوفوربيا) معروفة بخصائصها العلاجية. هدفت هذه الدراسة لتوضيح الخصائص الجزيئية لبعض انواع الجنس Euphorbia. ثلاث من هذه الانواع جمعت من ثلاث مناطق بولاية نهر النيل (*Euphorbia hirta, Euphorbia prostrata and Euphorbia milii*). التوصيف الجزيئي باستخدام تفاعل البلمرة المتسلسل و واسمات التضاعف العشوائي متعدد التشكل باستخدام 45 من البوادي اعطت نتائج للتضاعف وتعدد التشكل للنباتات قيد الدراسة.

كلمات مفتاحية: نبات ام لبينة، واسمات التضاعف العشوائي متعدد التشكل، تعدد التشكل

Introduction

The Euphorbiaceae exhibits a wide range of growth forms, extending from large xerophyte succulents to trees and small annuals (Dorsey, 2013). This variation in forms seems to be problematic for botanists and taxonomists due to the great variation in morphological features. As Webster (1994) stated, the major criteria for classifications in the past were morphological criteria of plant that can be physiologically or histologically identified. The taxonomic classification of Webster from 1994 was considered the most recommended systematic classification, however, difficulties in the classification of lower grades and their relationships in the Euphorbiaceae were encountered (Gilbert, 1994). Characteristic for the family Euphorbiaceae are the so called cyathia; mostly pink or greenish-yellow, single flower type formations, which represent inflorescences.

Molecular tools, developed in the recent years, were considered as an easy and relied-on mean for describing and differentiating plant taxa. They assisted to explain many taxonomic queries, which were not previously possible with only phenotypic methods. Each of molecular method has its own advantages and limitations. They differ in their determining strength to detect genetic differences, data they can provide and their applicability to help identifying taxonomic levels (Arif *et al*., 2010)**.**

The study aims to**:**

1- Identify the morphological characters of Genus Euphorbia.

2- Identify the species of Euphorbia using molecular analytical tools (the technique of RAPD-PCR).

Materials and methods

Sample collection

Leaves of studied plants were collected from different locations in River Nile State, Sudan (Alddamer, Atbara, Berber) on May-June 2016, washed, shaded in room temperature, stored in paper bags with silica gel for further use.

DNA extraction

From dried leaves, a sample of 0.5-1 gram were taken in falcon tube, 20 µl of CTAB (Hexadecyltrimethylammonium Bromide) added (used for breaking cell wall) to make a liquidated viscous paste, shacked by tissue layser (Kottermann Co.) in 20 rpm for 5-6 min, then transferred to revolving water bath (Lab company) with occasional mixing at 65C^{o} for 15-20 min. After cooling an equal volume (about 20 µl) of chloroform: isoamyl alcohol (24:1) added, transferred again to water bath, shacked every 2-5 min., then centrifuged at 14000 rpm (Arcelik Co.) for 10 min at 4C^{o}. After centrifugation supernatant was taken in new an eppendorf tubes. A volume of 500 µl of chilled Isopropanol (stored in -20C^{o}) or twice the volume from 95% Ethanol was added to it, inverted gently until two phases are no longer evident. DNA collected as white string mass, stored at -20C^{o} for 15-20 min or stored overnight at -20C^{o}. Centrifuged at 14000 rpm for 15 min at 4C^{o}, then supernatant discarded. Then 500µl of 76% Ethanol and 10 mM ammonium acetate were added to samples tubes. Then incubated at room temperature for 5 min. Ethanol ammonium acetate were withdrawn by pipette. Samples were left at room temperature in an air flow hood to dry. Then (TE) buffer added to dissolve the pellet of DNA. Stored at 4C^{o} for an overnight so that DNA dissolved. Next morning samples stored at -20C^{o} for further use (Adapted from Ahmad *et al*., 2004)

Estimation of the DNA concentration by spectrophotometer

A spectrophotometer (BIOTECH) was used to measure the optical density at wave length of 260 nm and 280 nm. Then 2 drops (correspond to approximately) 1µl were taken from DNA sample for measurement. The concentration of DNA was calculated according to the formula:

DNA concentration (µg⁄ml)= optical density 2 (µg⁄ml) ×50 ×Dilution factor.

The spectrophotometer was used also to measure the purity ratio according to this formula:

DNA purity ratio = optical density at 260 nm⁄ optical density at 280nm

The purity of DNA ratio was achieved to detect nucleic acid contamination with protein. The DNA quality can be also assessed by analyzing the DNA by agarose gel electrophoresis (Maniatis *et al*., 1982).

RAPD-PCR analysis protocol of genomic DNA of Euphorbia species

PCR was performed with a protocol includes the following:

Random primers

Forty-five primers, as indicated in Table (1), produced by Macrogen Co. in a lyopphizes form and were dissolved in sterile water to give a final concentration of 100 µM for stock and, 5µM for working sample as adapted from Yakoub (2006).

Table (1): Description of 45 iPBS primers with their name, sequences and annealing temperature (Ta)

Name	Sequence	Ta (°C)	Name	Sequence	Ta (°C)
2074	GCTCTGATACCA	50	2391	ATCTGTCAGCCA	52
2075	CTCATGATGCCA	50	2392	TAGATGGTGCCA	52
2246	ACTAGGCTCTGTATACCA	50	2399	AAACTGGCAACGGCGCCA	52
2255	GCGTGTGCTCTCATACCA	50	2085	ATGCCGATACCA	53
2257	CTCTCAATGAAAGCACCA	50	2095	GCTCGGATACCA	53
2380	CAACCTGATCCA	50	2228	CATTGGCTCTTGATACCA	53
2381	GTCCATCTTCCA	50	2230	TCTAGGCGTCTGATACCA	53
2382	TGTTGGCTTCCA	50	2251	GAACAGGCGATGATACCA	53
2385	CCATTGGGTCCA	50	2374	CCCAGCAAACCA	53
2393	TACGGTACGCCA	50	2377	ACGAAGGGACCA	53
2400	CCCCTCCTTCTAGCGCCA	50	2383	GCATGGCCTCCA	53
2402	TCTAAGCTCTTGATACCA	50	2077	CTCACGATGCCA	55
2249	AACCGACCTCTGATACCA	51	2232	AGAGAGGCTCGGATACCA	55
2273	GCTCATCATGCCA	51	2237	CCCCTACCTGGCGTGCCA	55
2398	GAACCCTTGCCGATACCA	51	2238	ACCTAGCTCATGATGCCA	55
2229	CGACCTGTTCTGATACCA	52	2239	ACCTAGGCTCGGATGCCA	55
2231	ACTTGGATGCTGATACCA	52	2272	GGCTCAGATGCCA	55
2252	TCATGGCTCATGATACCA	52	2373	GCTCATCATGCCA	55
2276	ACCTCTGATACCA	52	2390	GCAACAACCCCA	55
2277	GGCGATGATACCA	52	2079	AGGTGGGCGCCA	63
2375	TCGCATCAACCA	52	2081	GCAACGGCGCCA	63
2376	TAGATGGCACCA	52	2274	ATGGTGGGCGCCA	63
2387	GCGCAATACCCA	52			

Amplification reaction

A total volume of 25 µl was used to run PCR amplification reaction. Exactly 12.5 µl of the PCR ready mix (containing 0.2µl of dream taq polymerase, reaction buffer and $MgCl_2$). Sterile distilled water was used to achieve a total volume of 25 µl after adding of each of dNTP primers and DNA template. Amplification of random fragments of genomic DNA was performed with the following master amplification reaction (Table 2).

Table (2): PCR Program

The amplification program was run as follow using thermo cycler:

Step -1 Initial –denaturation	95°C	3 min
30 cycles		
Step-2 Denaturation	95°C	15 Sec
Step-3 Annealing	XX°C	60 Sec
Step-4 Extension	72°C	2 min
Step-5 Final extension	72C°	7 min

xx =temperature degree of the primer

Approximately 25µl of PCR amplified products were separated by electrophoresis in 1.8% agarose gels (90min,105V/cm, 0.5X Tris-borate buffer). Gels stained with ethidium bromide (12µl), PCR products were visualized by U.V transilluminator (Minilumi) and then were imaged by gel documentation system (Bio-RAD) as adapted from Hashemi *et al*. (2009). The size of RAPD-PCR products estimated by reference marker 1Kb DNA ladder 100-10,000 bp.

Molecular weight markers

The DNA markers solution (100-3000bp), thermo gene ruler mix (100bp-10000bp) were used both when PCR product were visualized, in DNA extraction Solis (100-3000) bp was used only.

Agarose gel electrophoresis for DNA extraction

Checking DNA concentration on gel:

To check 1-9 samples making mixture of (ddH_2O-Bule dye) (Master mix should be for 11 samples):

ddH_2O =11× 8µl =88 µl

Blue dye =11× 2µl = 22µl

The total volume was 110, 10µl. for the mixture, 20µl from DNA samples were added to each tube of mixture so as to be ready for running Electrophoresis voltage =75v in 30 min.

Statistical analysis

Genotyping results were computed as polymorphism percentage based on polymorphic bands compared to total number of bands to obtain Shannon's information index.

Binary coding technique, where presence or absence of band denoted by (1) and (0), and data generated were fed to the software. Genetic similarity indexes, based on Jaccard coefficient, was then obtained using PAST 3.14 computer software.

Results and discussion

DNA Extraction from dry leaves of Euphorbia species

The extraction of genomic DNA from dry leaves of *Euphorbia spp*. using CTAB produced good quality (ratio 1:1.5) and high purity of intact DNA to be used in the RAPD-PCR analysis. The purity of the extracted DNA yield of *E.hirta, E.prostrata, E.milii* (Alddamar, Atbara, Berber) were 1.94, 1.82, 1.93, 1.57, 1.53, 1.15, 1.33, 1.72 and 1.44, respectively as checked by spectrophotometer. The integrity of the extracted DNA checked by agarose gel (1%).

The DNA samples extracted from dry leaves were very stable and could be stored in 4 to -20 °C for a long time without degradation, so it could be used in further studies. These results resemble that obtained by Ahmadikhah and Alvi (2009).

RAPD-PCR Analysis

RAPD-PCR technique usually used to express polymorphism in DNA of the studied *Euphorbia spp*. in order to search for the sources of differences that could be used as a DNA marker.

The primers used in the study were selected randomly, 45 primers had been tested with same DNA samples under optimum conditions.

As shown in Table (3), 798 out of the total of 819 bands were scored as polymorphic representing 97.4%. That means there is high differences among genotype of the species of *Euphorbia*. The analysis of PCR amplified DNA fragments relies on several bases including the absence or presence of bands, difference in molecular weight and distinct divergence in intensity of bands, which in this study, was not taken much into account due to the presence of obvious differences in total number of bands and their molecular weight among study species.

Primer (2237) produced the highest number of polymorphic bands (33), while, the minimum number (one) was generated using primer (2231). The average number of polymorphic bands per primer among the species was (17.73). Polymorphism of each primer was calculated as the percentage of polymorphic bands to the number of a total main bands produced by the designated primer. This result of high polymorphism rate indicates a high genetic diversity between species with respect to geographical location and species wise.

The variation in the number of bands amplified by different primer influenced by variable factors such as primer structure, template quantity and less number of annealing sites in the genome (Kernodle *et al*., 1993).

In RAPD-PCR analysis, the difference in the number of main and other amplified bands is mainly due to the primer structure and that some primers recognize a large number of link locations, which are more useful than those recognizing a lower number of these locations, giving better chance of detecting DNA polymorphisms among individuals.

Sources of polymorphism as reflected on gel may include detection of priming site, insertion that render priming site too distant to support amplification or insertions that change the size of a DNA segments without preventing its amplification. Furthermore, it had been reported that single nucleotide change in a primer sequence may cause a complete change in the pattern of amplified DNA segments (William *et al*., 1990; Devos and Gale, 1992).

Visual examination of gels electrophoresis and analysis of banding pattern confirmed that *E herita* in the three geographical location were closer to each other compared to the two other species (Table 5 and Fig 1). *E. prostrata* Alddamar and *E. milii* Alddamar had degree of similarity appeared in the pattern of DNA with most of primers. Similarity between *E. prostrata* Atbara and *E. prostrata* Berber, in another way, indicated by genotyping results (Table 5) and interfered by clear differences among them specially in term of unique bands (Table 4). Meanwhile, *E. milii* Atbara had less similarity with all others, location and species wise (*E milii* Berber produced DNA but failed in amplification to be used for comparison).

The RAPD assay with the protocol used, as taxonomic tool, generated well discriminating products in 8 of the species studied. Thus may be used as DNA fingerprints for species identification. It would be of huge use for the determination of species distinction. On the other hand, RAPD markers had been useful as the first step to produce a genetic map in plants with less known genetic series (Sesli and Yegenoglu, 2017). Results confirm the relationship between the three species of *Euphorbia* obviously; as well as distinction of *E. milii* from the other two species (Tables 4), and this corresponds to the morphological features of these species. Then RAPD could be applied in differentiation between species even if morphological characters could not be

easily used as stated by Williams *et al*. (1990). This is one of the prime goals of this bio systematic study which can use more than one method in the diagnosis of the rest of *Euphorbia* species existing in Sudan.

Table (3): Genotyping results for IPBS marker

Primer	BS (bp)	TBN	PBN	PP (%)	PIC	*H*	*I*
2382	250 – 3000	20	20	100,0	0,42	0,36	0,54
2074	575 – 3000	24	24	100,0	0,36	0,31	0,47
2393	300 – 2500	17	17	100,0	0,32	0,20	0,35
2276	500 – 2500	3	3	100,0	0,22	0,12	0,24
2375	180 – 3000	25	25	100,0	0,37	0,28	0,44
2399	300 – 3000	23	23	100,0	0,39	0,31	0,47
2251	350 – 3000	22	19	86,4	0,33	0,32	0,47
2232	250 – 3000	29	29	100,0	0,36	0,33	0,49
2373	300 – 3000	16	16	100,0	0,36	0,26	0,42
2257	480 – 3000	9	9	100,0	0,34	0,23	0,39
2075	275 – 3000	20	20	100,0	0,37	0,26	0,42
2246	1000 – 3000	5	5	100,0	0,25	0,14	0,27
2277	220 – 3000	33	33	100,0	0,35	0,24	0,40
2376	230- 3000	31	31	100,0	0,35	0,28	0,44
2085	410 – 2600	11	11	100,0	0,30	0,19	0,33
2374	170 – 3000	29	29	100,0	0,34	0,30	0,46
2237	180 – 3000	33	33	100,0	0,33	0,23	0,38
2390	150 – 3000	20	20	100,0	0,39	0,29	0,46
2255	900 – 3000	8	8	100,0	0,34	0,23	0,39
2380	280 – 3000	20	20	100,0	0,36	0,25	0,41
2249	400 – 3000	17	17	100,0	0,39	0,33	0,50
2387	550 – 3000	21	20	95,2	0,33	0,24	0,39
2095	300 – 3000	22	22	100,0	0,32	0,21	0,36
2377	350 – 3000	21	21	100,0	0,30	0,19	0,32
2238	520 – 3000	14	14	100,0	0,36	0,24	0,40
2079	150 – 3000	25	20	80,0	0,29	0,29	0,43
2400	300 – 3000	15	15	100,0	0,43	0,35	0,52
2381	180 – 3000	16	16	100,0	0,29	0,21	0,35
2273	250 – 3000	15	15	100,0	0,27	0,16	0,29
2231	2000	1	1	100,0	0,47	0,33	0,51
2391	280-3000	23	22	95,7	0,38	0,28	0,44
2228	475 – 3000	20	19	95,0	0,37	0,26	0,41
2383	80 – 3000	22	18	81,8	0,32	0,27	0,41
2239	120 – 3000	24	24	100,0	0,37	0,31	0,48
2081	180 – 3000	24	20	83,3	0,30	0,23	0,36
2402	1100 – 3000	6	6	100,0	0,27	0,17	0,30
2085	410 – 2600	11	11	100,0	0,30	0,19	0,33
2398	490 – 2400	5	5	100,0	0,27	0,16	0,29
2252	530 – 3000	6	6	100,0	0,29	0,17	0,31
2392	250 – 3000	19	19	100,0	0,39	0,32	0,49
2230	350 – 3000	21	21	100,0	0,38	0,26	0,43
2077	150 – 3000	21	20	95,2	0,36	0,31	0,47
2272	360 – 3000	21	21	100,0	0,29	0,18	0,32
2274	150 – 3000	17	16	94,1	0,35	0,32	0,48
2229	900 – 3000	14	14	100,0	0,34	0,23	0,39
Total	**-**	**819**	**798**	**97,4**			
Mean					**0,34**	**0,25**	**0,40**

BS: band sizes; **TBN**: total band number; **PBN**: polymorphic band number; **PP**: polymorphism percentage; **PIC**: average polymorphism information content; ***H***: gene diversity; ***I***: Shannon's information index

Table (4): Unique bands generated from designated primers with different Euphorbia spp.

Primer	Molecular wt. of unique bands							
	E.hirta, **Alddamer**	***E.hirta*** **Atbara**	***E.hirta,*** ***Berber***	***E.prostrata*** **Alddamer**	***E.prostrata*** **Atbara**	***E.prostrata*** **Berber**	**E.** ***milii*** **Alddamer**	**E.** ***milii*** **Atbara**
2075				1600 800			2000 780	1750
2382		850		800 250			500	
2400					450			
2249				750			890 400	
2375				2500 950 590 280			880 740 450	920
2387				1750	870 550		1900 980 750	
2391		1080 750 600		470				
2392		1550		250		1500		
2228				500		1000 950	470	
2230		600 400		500 350				
2251				350		750	600	
2374		850 300			420		1050 850 360 320 170	180
2383				280 200 80				
2077			700 660 300	250			400 150	
2390				250 150			800	
2079				300				
2081			690	490 390 360				
2274		1000		790	830			

Table (5): Genetic similarity indexes based on Jaccard's coefficient using PAST 3.14 software

Species	1	2	3	4	5	6	7	8
1	1							
2	0.43	1						
3	0.52	0.45	1					
4	0.19	0.32	0.23	1				
5	0.21	0.29	0.24	0.34	1			
6	0.20	0.27	0.23	0.33	0.57	1		
7	0.20	0.27	0.21	0.38	0.28	0.28	1	
8	0.20	0.18	0.17	0.20	0.23	0.22	0.26	1

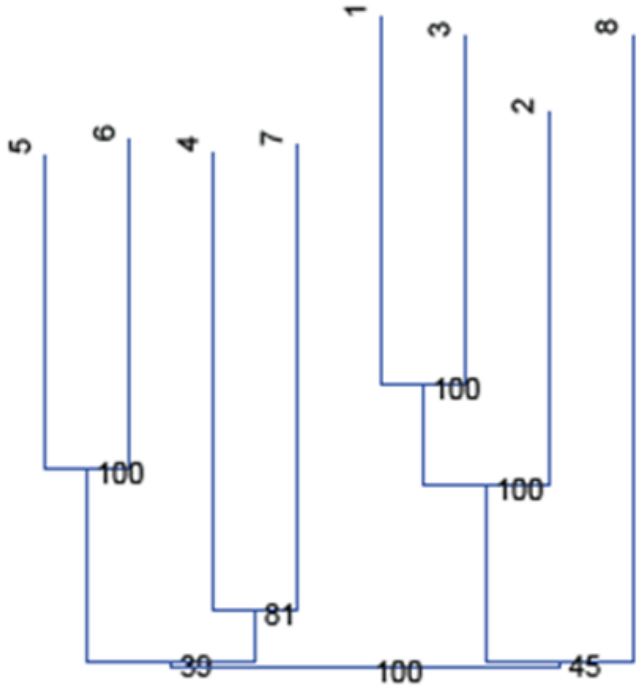

Fig. (1): Phylogenic Tree: 1. E.hirta (Alddamar), 2. E.hirta (Atbara), 3. E.hirta (Berber), 4. E.prostrata (Alddamar), 5. E.prostrata (Atbara), 6. E. prostrata (Berber), 7. E.milii (Alddamar), 8. E.milii (Atbara).

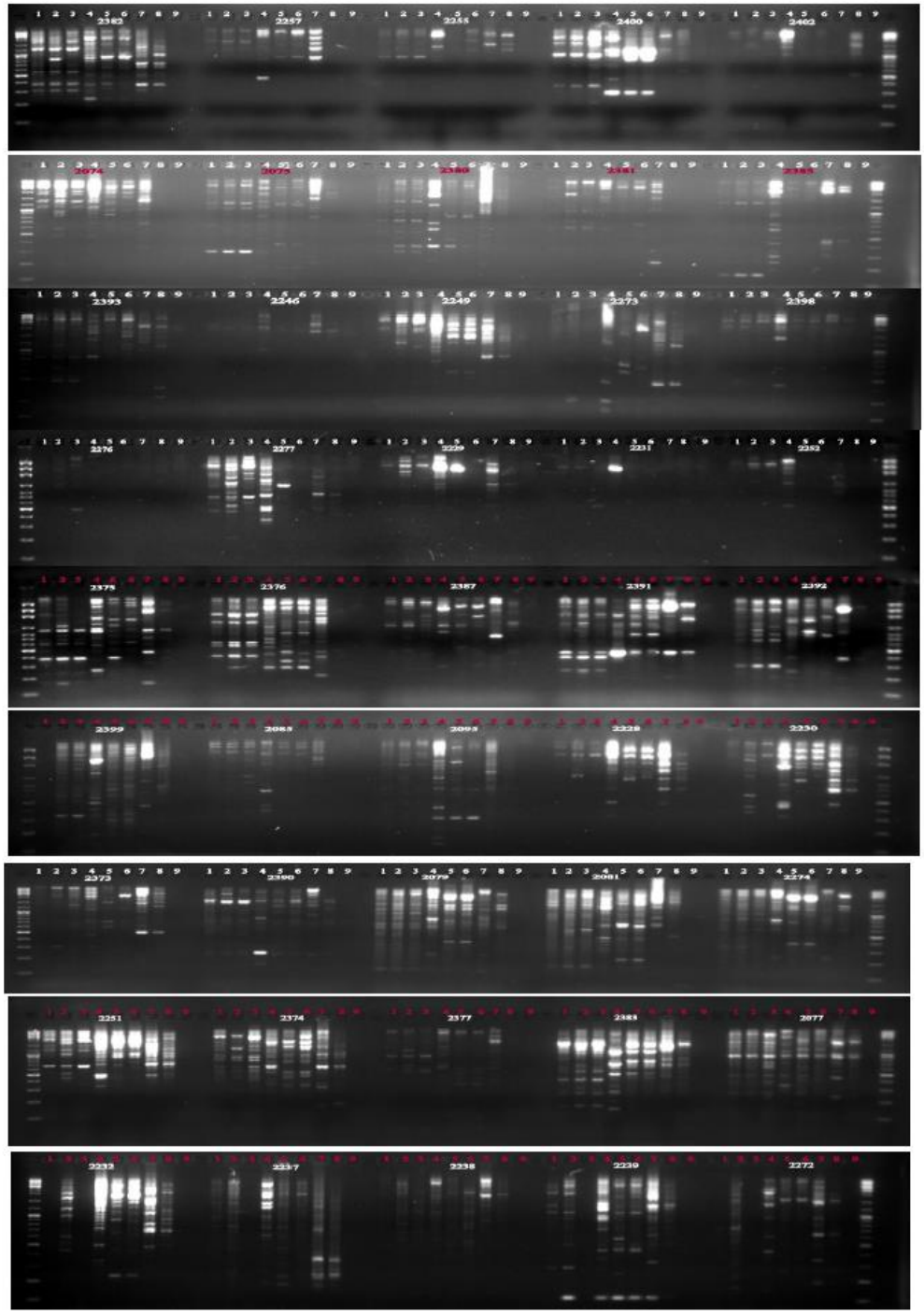

Fig. (2). Visualized Primer results

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