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Original Article

MOLECULAR CHARACTERIZATION OF DATE PALM (*PHOENIX DACTYLIFERA*) USING COMBINED MARKER ANALYSIS GROWN IN KUTCH REGION OF INDIA

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Abstract: In Date palm (Phoenix dactylifera L.) molecular characterization to find the best genotype and male/female identification at an early stage before flowering is of great economic significance to the farmers. Genetic finger printing using molecular markers is an important tool. In present investigation four types of markers RAPD, ISSR, SSR, ITS gene were applied in 5 unknown genotypes of Date palm (female 1, female 2, female 3, Male 2 small and Male1 tall). Four type of markers (highly polymorphic) with two best primers for each marker (RAPD, ISSR, SSR and ITS), were used. Amplification of genomic DNA of 5 Date palm genotypes yielded 32 scorable fragments out of which 17 were polymorphic, with an average of 4.25 polymorphic fragments per primer. Number of amplified fragments ranged from 3 (BNL-448) to 10 (OPG-2 and ITS-3) and varied from ~200bp to ~4kb. Percentage of polymorphism ranged from 20% (ITS-3) to a maximum of 100% (BNL-448). Cluster analysis using UPGMA contains two clusters A and B with Jaccard's similarity matrix with low value of 0.660 in that female 2 genotype was distinct. On the other hand, cluster A contains female 3 and Male 1 tall with highest value of 0.810 and Male 2 small genotype having similarity value of 0.790. In cluster A1 female 1 genotype with similarity value of 0.750 was found diverse in cluster A.

Key words: Date Palm, Markers (RAPD, ISSR, SSR, ITS)

INTRODUCTION

The Date palm (*Phoenix dactylifera* L., 2n=36) is a dioecious long-lived monocotyledon plant, which belongs to family Palmaceae. Date is commercially grown in Saudi Arabia, Iran, Iraq, Libya, Egypt, Morocco, Tunisia, Sudan, Algeria, Pakistan, India, USA, and Spain and plays important role in the economic development of these countries. Total production of date palm fruit in world ranges between 2.5 and 4 million tons per year. If we look at the distribution region we find that Asia is in the first position with 60 million Date palms per year.

Date palm is one of the important horticultural crops of the Kutch region which enjoy the monopoly of commercial cultivation of this crop in India. The heterogeneous genetic form of Date palm makes its seed raised progeny strongly heterogeneous and variable and, therefore, multiplication of Date palm is mainly done vegetatively. Characterization and analysis of the available genetic diversity therefore, constitute indispensable step with regard to the development of breeding strategies.

Biochemical markers (isozymes and proteins) have proven to be effective in varietal identification as well [1,2]. However, they give limited information and are usually indirect approach for detecting genomic variation. The molecular markers are useful tools for identification and phylogenetic analysis of different plant species and cultivars. Genetic finger printing

using molecular markers have many important applications, including germplasm conservation, assessment of seed purity and verification of labelling and identify plant in production and marketing [3]. In addition, the characterization of cultivars and evaluation of genetic diversity require a large set of phenotypic data that normally is difficult to collect and statically variable due to environmental effects [4]. DNA markers have also been applied as a direct approach to detect genetic variation and sex determination in Date palm genotypes [5].

There is tremendous variation in the genotypes grown in Kutch. Because of the seeds thrown by the army camps, partly from the seeds and offshoots planted by the settlers and due to cross-pollination mode of reproduction. Therefore, it is urgent need to document these genotypes and find out characteristics of the best genotype growing under Kutch conditions in order to expand/replace cultivar/s for higher and quality yield. Another problem for farmers is to distinguish male and female at early stages before flowering. Identification of male/female at an early stage would save farmers from heavy losses due to unproductiveness of male plants.

MATERIALS AND METHODS

Plant material: Leaf samples of Date palm germplasm were collected from Kutch district of Gujarat. It included five Date Palm unknown genotypes with two male namely Male 1 tall and Male 2 small and three female genotypes Female 1, Female 2 and Female 3 with different characters.

DNA extraction: Total Genomic DNA of eight Date palm genotypes was extracted from 500 mg leaf samples using modified CTAB (Cetyl Trimethyl Ammonium Bromide) method of Doyle and Doyle [6with few modifications. The leaves were first grind in to a fine powder in liquid nitrogen using autoclaved and pre-chilled mortar and pestle. It was then transferred in to a pre-warmed extraction buffer (1.5M Nacl, 100mM Tris-Hcl pH-8, 40mM EDTA, 1% PVP, 3% C-Tab and 1% â-mercaptoethanol.) and incubated at 65°C for 1hr. Equal volume of chloroform : isoamyl alcohol (24:1 v/v) was added, mix well by gentle inversion and centrifuged. The supernatant was transferred to a fresh tube and DNA was precipitated by adding 0.6 volume of ice-cold isopropanol. After centrifugation, the pellet was washed in 70% ethanol, dried and dissolved in TE buffer. RNA was removed by RNase treatment. DNA was quantified using UV-Spectrophotometer and diluted to 50-60 ng/µl and used in PCR.

Selection of primer: Four RAPD primers which are highly polymorphic were chosen as potentially useful, for combined marker analysis from fifteen, 10-mer oligonucleotides with arbitrary sequence. Ten primers based on dinucleotide repeats were used in ISSR analysis. Two ISSR primers that produced clear and reproducible fragments were selected for the amplification of all DNA samples. Ten primers pairs were tested with temperature gradient running from 45°C to 60°C. From this two primers were selected for full analysis. Four ITS primers were screened out of which two were selected for polymorphic banding patterns.

Molecular analysis: PCR (Polymerase chain reaction) for both RAPD and ISSR analysis was performed in 20µl volume containing 1x PCR buffer (Banglore Genei), 1.5 mM $MgCl_2$, 0.2 mM dNTPS, 0.1µM of primer, 50ng of genomic DNA and 1 U Taq DNA polymerase (Banglore Genei). The reaction mixture was placed on DNA thermal cycler (Biometra). RAPD program was performed as 1 cycle of 94°C for 5 min and 40 cycles of 94°C for 1 min, 38°C for 1 min and 72°C for 2 min, then, a final extension step 72°C for 8 min. The ISSR program was performed as 1 cycle of 94°C for 5 min and 35 cycles of 94°C for 1 min, 45°C for 45 sec, 72° C for 1.5 min and a final extension step of 72°C for 8 min. The performed ITS program was the same as that of RAPD. The SSR program was performed as 1 cycle of 95°C for 5 min and 35 cycles of 95°C for 30 sec, 52°C for 1 min, 72° C for 1 min and a final extension step of 72°C for 7 min. PCR products were electrophoreses on 1.5% (w/v) agarose gels, in 0.5X TBE Buffer at 80 V for 1 hr for RAPD, ISSR and ITS while PCR product of SSR were separated in 8% native PAGE and then stained with ethidium bromide (0.5µg/ml). Gels with amplified fragments were visualized and photographed under UV light.

Scoring and Data analysis: DNA bands were scored for computer analysis on the basis of presence or absence. If a product was present in a genotype it was designated “1”, if absent it was designated “0” after excluding irreproducible bands. Pair-wise comparisons of genotypes, based on presence or absence of unique and shared polymorphic products, were used to generate similarity coefficients based

on similarity matching. Using distance matrix a principal coordinate analysis to construct a three-dimensional array of eigenvectors was performed using DCENTER module of NTSYSpc 2.2 program.

RESULTS AND DISCUSSION

Combined banding Patterns of RAPD, ISSR, SSR and ITS markers: Five Date palm genotypes namely Female 1, Female 2, Female 3, Male-1tall and Male-2 small as obtained from kutch district of Gujarat were amplified using selected best primer each from RAPD, ISSR, SSR and ITS oligonucleotide. Amplification of genomic DNA yielded 128 fragments that could be scored.

Amplification products of 5 genotypes with four best selected RAPD primers are OPF-2, OPF-4, OPG-2 and OPL-8 (Table 1). In which OPG-2 yielded a total of 10 scorable bands, out of which 8 (80.00%) and OPF-4 yielded a total of 6 scorable bands, out of which 5 (83.33%) were found to be polymorphic. While primer OPF-2 contains a total of 5 scorable bands, out of which 3 (60.00%) were found to be polymorphic and primer OPL-8 contains a total of 5 scorable bands, out of that 3 (60.00%) were found to be polymorphic. The size of the amplification product ranged from 200bp to 4000bp. Using 19 RAPD primers Sedra et al. [4 also observed 66.07% polymorphism among 43 date palm accessions. In figure 1 using RAPD primer OPG-2 showed amplification banding pattern of 10 scorable bands ranging in size from ~220bp to ~900bp. Two bands of size ~400bp and ~700bp were monomorhic bands. A band of ~650 size was unique to genotype female 2 (lane-2) while absent in other 4 genotypes. Five bands of sizes ~220bp, ~300bp, ~850bp, ~900bp and 920bp were absent in female 2 genotype while present in other four genotypes. A band of size ~870bp was present in female 1 and 2 while absent in other three genotypes.

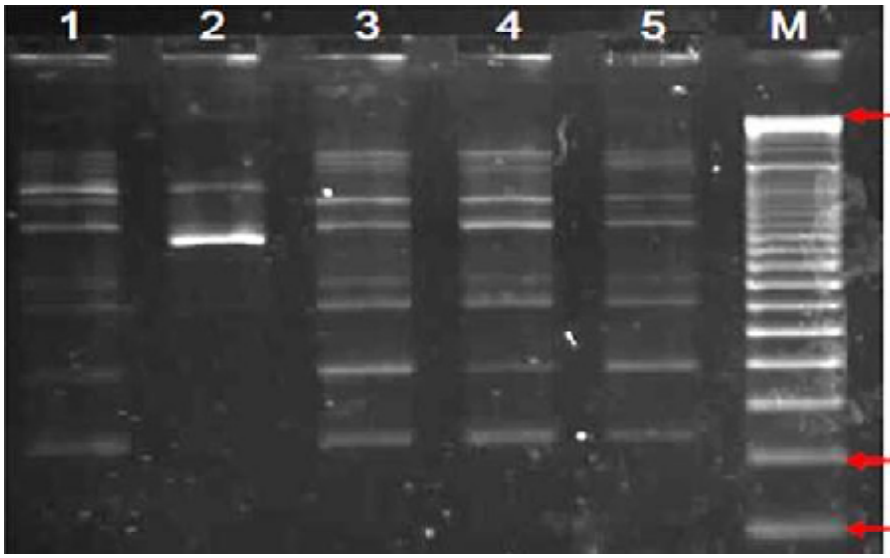

Fig. 1: RAPD amplification pattern of 5 Date palm genotypes using primer OPG-2. Lane 1-3 are genotypes of Female 1, 2 and 3 while, lane 4 and 5 contains Male 2 tall and Male 1 small

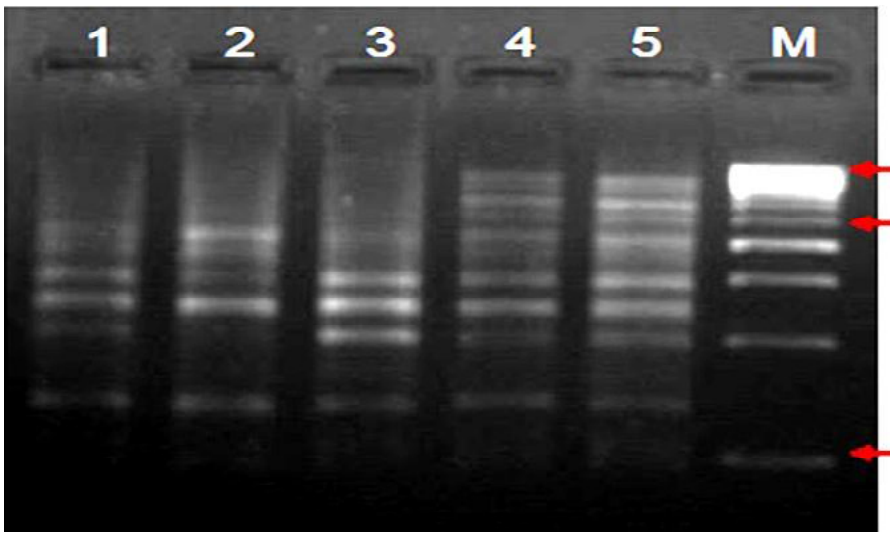

Fig. 2: ISSR amplification pattern of 5 Date palm genotypes using primer UBC-817. Lane 1-3 are genotypes of Female 1, 2 and 3 while, lane 4 and 5 contains Male 2 tall and Male 1 small

Table 1: Details of amplification obtained with different RAPD primers

Sr. No.	Primer	Sequence (5'—3')	No. of total bands	No. of Polymorphic bands	No. of Monomorphic bands	Polymorphism percent (P%)	Total No. of bands amplified
1.	RAPD OPG-2	GGCACTGAGG	10	8	2	80.00	37
2.	RAPD OPF-2	GAGGATCCCT	5	3	2	60.00	17
3.	RAPD OPF-4	GGTGATCAGG	6	5	1	83.33	22
4.	RAPD OPL-8	AGCAGGTGGA	5	3	2	60.00	19
Total			32	17	12	53.13	128

Table 2: Details of amplification obtained with different ISSR primers

Sr. No.	Primer	Sequence (5'—3')	No. of total bands	No. of Polymorphic bands	No. of Monomorphic bands	Polymorphism percent (P%)	Total No. of bands amplified
1.	ISSR UBC-817	CACACACACA CACACAA	9	5	4	55.55	35
2.	ISSR UBC-827	TCTCTCTCT CTCTCTCRG	9	2	7	22.22	43
Total			18	7	11	38.88	78

Table 3: Details of amplification obtained with different SSR primers

Sr. No.	Primer	Sequence (5'—3')	No. of total bands	No. of Polymorphic bands	No. of Monomorphic bands	Polymorphism percent (P%)	Total No. of bands amplified
1.	SSR BNL-448	F-GCAGCTTGCTTGCA AGCTTC R-ACGCAAGCTTGGTC-AATACC	5	3	2	60.00	16
2.	SSR BNL-827	F-GCAAGGCAAGAAG CGCGC R-CGCTAGCATGCTAG-CACG	6	2	3	33.33	26
Total			11	5	5	45.45	42

Table 4: Details of amplification obtained with different ITS primers

Sr. No.	Primer	Sequence (5'—3')	No. of total bands	No. of Polymorphic bands	No. of Monomorphic bands	Polymorphism percent (P%)	Total No. of bands amplified
1.	ITS-3	GCATCGATGAAGAA-CGCAGC	10	2	8	20	45
2.	ITS-5	GGAAGTAAAAGTCG-TAACAAGG	5	1	4	20	23
Total			15	3	12	20	68

Amplification products of 5 genotypes with two best selected ISSR primers are UBC-817 and UBC-827 (Table 2). Primer UBC-817 yielded a total of 9 scorable bands, out of which 5 (55.55%) were found to be polymorphic while, UBC-827 contains 9 scorable bands, out of which 2 (22.22%) were found to be polymorphic. The size of the amplification product ranged from 300bp to 4000bp and the primer used was UBC-817. While Zehdi et al. [7] observed genetic similarity coefficient ranged from 0.30 to 0.78 with polymorphism of 79.38% using 12 primers among 12 Date palm genotypes. ISSR marker UBC-817 had amplified 9 bands ranging in size from 750bp to 4kb with 5 monomorphic bands ranging in size from ~750bp to 1.5 kb. A band of size 1kb was absent in female 2 genotype while present in other four genotypes.

Amplification products of 5 genotypes with these two best amplified SSR primers BNL-448 and BNL-827 (Table 3). BNL-448 yielded a total of 5 scorable bands, out of which 4 (80%) were found to be polymorphic while, BNL-827 contains total of 6 scorable bands, out of which 2 (33.33%) bands were found to be polymorphic. The size of the amplification product ranged from 300bp to 4000bp and the primer used was BNL-448. Ahmed et al. [8] had analyzed genetic diversity among 15 different cultivars of Date

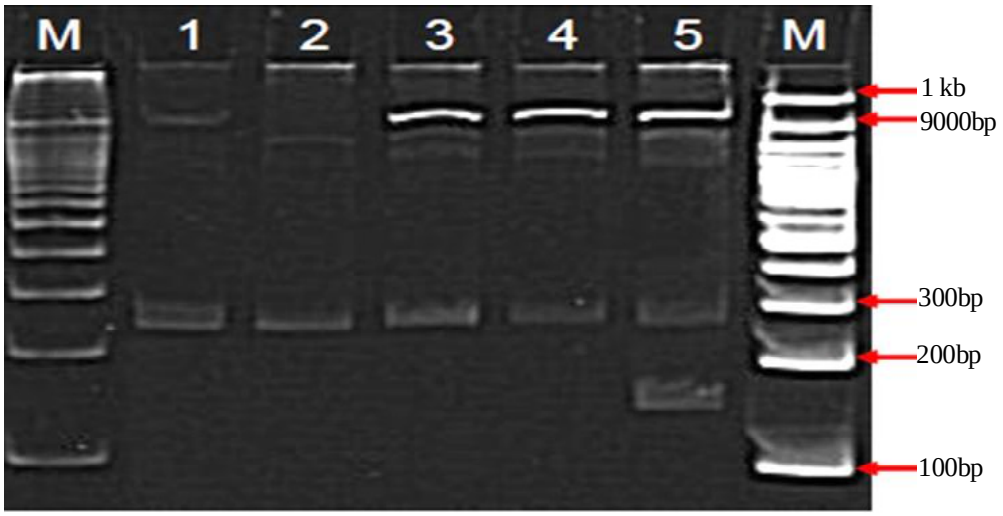

Fig. 3: SSR amplification pattern of 5 Date palm genotypes using primer BNL-448. Lane 1-3 are genotypes of Female 1, 2 and 3 while, lane 4 and 5 contains Male 2 tall and Male 1 small

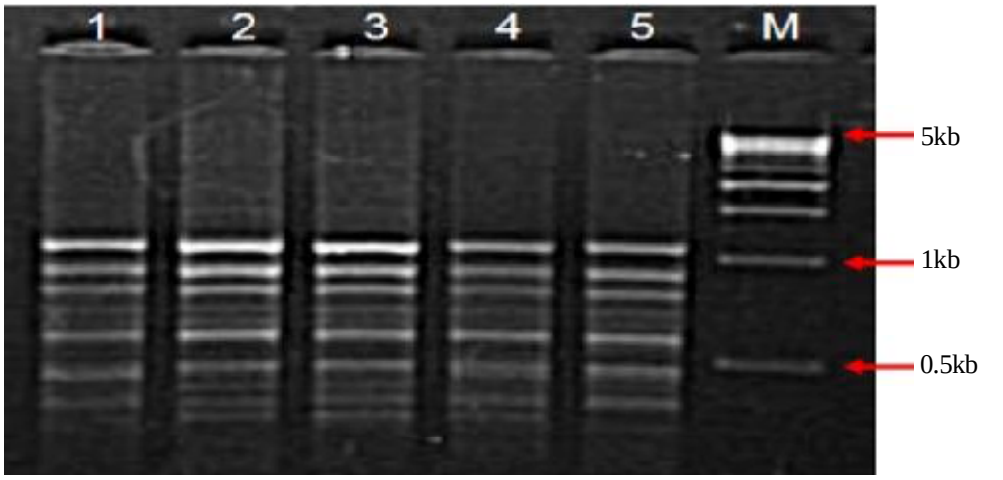

Fig. 4: ITS amplification pattern of 5 Date palm genotypes using primer ITS-3. Lane 1-3 are genotypes of Female 1, 2 and 3 while, lane 4 and 5 contains Male 2 tall and Male 1 small

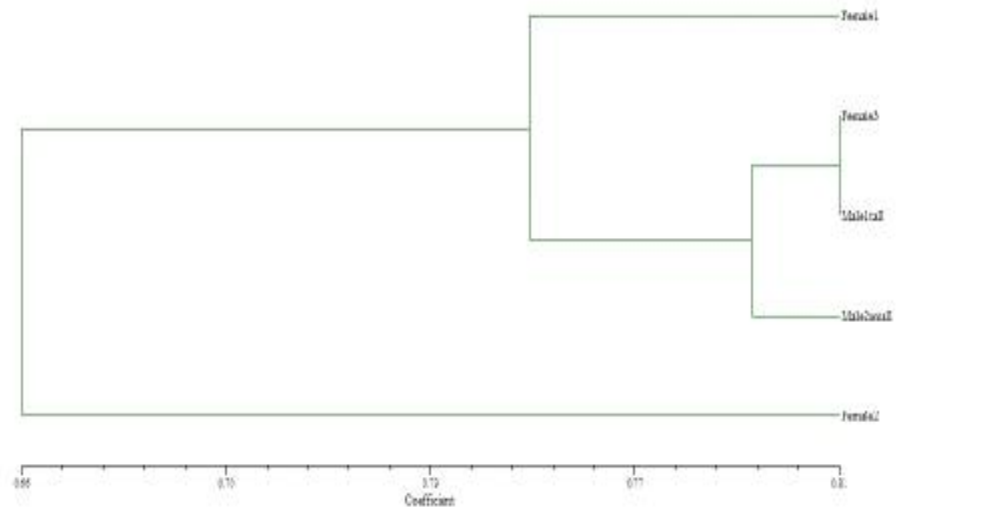

Fig. 5: .Dendrogram depicting the genetic relationship among 5 date palm genotypes consisting of of Female 1, 2 and 3 while, lane 4 and 5 contains Male 2 tall and Male 1 small

palm. Sixteen primers were tested for their ability to generate expected SSR banding patterns of which 10 primers successfully produced clear single bands in most of studied genotypes. Amplified SSR band sizes ranged from 100-300 bp. SSR marker BNL-448 had revealed three scorable bands ranging in size from ~840bp to ~950bp. A band of ~840bp was present in genotype female 3, male 2 small and male 1 tall (lane 3, 4 and 5) while absent in other two genotypes. A band of size ~900bp and ~950bp were unique to female 1 and female 2, respectively, while absent in other four varieties.

Amplification products of 5 genotypes with two ITS primers are ITS-3 and ITS-5 (Table 4). ITS-3 yielded a total of 10 scorable bands, out of which 2 (20%) were found to be polymorphic while, ITS-5 contain 5 scorable bands in which 1 (20%) band was found to be polymorphic. The size of the amplification product ranged from 300bp to 4000bp and the primer used was ITS-3. Ahmed et al. [9] analyzed percentage of genetic similarity between Egyptian males and females Date palms at DNA level by PCR using random primers. RAPD technique was applied using five primers of 10 mers and one of 20 mers. The result showed highest similarity percentage with males 1, 2 and 3 genetically closely related to Malkabi, Bartamoda, Sakkoty and Dagana cultivars (91.2, 86.5, 81.2 & 79.0 %, respectively). Out of ten scorable bands of ITS-3, eight were monomorphic bands. A band of ~350bp size was present in female 2, female 3 and male 2 small while absent in Ghanshyam and male 1 tall. A band of ~450bp size was present in female 2 and female 3 while absent in other three genotypes.

Combined dendrogram analysis using RAPD, ISSR, SSR and ITS: Cluster analysis using UPGMA contains two clusters A and B with Jaccard's similarity matrix with low value of 0.660 of female 2 genotype was group in to distinct cluster B. On the other hand, cluster A contains two subclusters A1 and A2. In cluster A1 female 1 genotype with similarity value of 0.750 was found diverse in cluster A. While cluster A2 contains the genotypes female 3 and male 1 tall with highest value of 0.810 and male 2 small genotype having similarity value of 0.790. In contrast, Haider et al. [10] observed higher polymorphism in 23 date palm cultivars from Syria representing 18 female and five male cultivars. The average polymorphism detected by the RAPD assay (58.5%) was higher than that observed for ISSR (50.6%). These results might be due to more number of genotypes and primers were used in study.

CONCLUSION

From the above study it can be concluded that RAPD markers revealed sufficient genetic diversity with more than 80% polymorphism than ISSR, SSR and ITS markers. In ISSR marker not much genetic diversity was recorded but some male specific unique bands were noticed. This may be attributed to the use of very less number of markers in the study. There is need to verify the results with RAPD and ISSR marker with more number of primers as well as genotypes. In RAPD and ISSR combined marker data, the UPGMA dendrogram obtained were with almost the same sample distribution pattern. On the other hand, dendrogram from ISSR high similarity matrix value suggesting that RAPD was more efficient than ISSR in Date palm.

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Abbreviations used: RAPD = Random Amplified Polymorphic DNA, ISSR = Inter Simple Sequence Repeats, SSR =Simple Sequence Repeats, ITS = Internal ranscribed Spacer, UPGMA = Unweighted Pair Group Method with Arithmetic Mean. BNL =Brookhaven National Laboratory, OPG = Operon Technologies, CTAB = Cetyl Trimethyl Ammonium Bromide, PCR = Polymerase chain reaction,

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