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EFFECT OF RELATIVE MOBILITY OF RAPD-PCR FRAGMENTS ON DATA DEVIATION.

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ABSTRACT

The results give evidence that, the proper choice of electrophoretic mobility (greater extend or smaller extend) of PCR fragments according to:

I- At emerge of strong amplification of PCR product and intense fragments from agarose well to gel matrix; this condition need to greater extend (long R_m).

II- At emerge of poor amplification of PCR product and faint fragments from agarose well to gel matrix, this condition need to smaller extend (small R_m).

Maximizing the number of fragments by greater extend in the case of strong amplification and keeping of the stability of the number of fragments by smaller extend in case of poor amplification is the suitable choosing for R_m. of RAPD-PCR fragments. The previous condition will increase the characters (units) which used in Operational Taxonomic Units (OTUs) to give more quantity and quality differences among tested variables. Additional analysis can then be undertaken on the dendrogram to assess the distinctness and validity of clusters, and constant characters for later use in diagnostic schemes can be determined.

The choice of electrophoretic mobility becomes a methodological factor to obtain optimal resolution, discriminate the cryptic data, give unique patterns and avoid the deviation in data. Eculidian distance, Size differences, Lambada and Anderberg,sD cluster analysis are used in intraspesific variation.

INTRODUCTION

DNA molecules, like proteins and many other biological compounds, carry an electric charge, negative in the case of DNA. Consequently, when DNA molecules are placed in an electric field they will migrate towards the positive pole. The rate of migration of a molecule depends on two factors, its shape and its electric charge. Unfortunately, most DNA molecules are the same shape and all have very similar electric charges.

However, the size of the DNA molecule does become a factor if the electrophoresis is performed in a gel, which is usually made of agarose, polyacrylamide or a mixture of the two, comprises a complex network of pores, through which the DNA molecules must travel to reach the positive electrode. The smaller the DNA molecule, the faster can migrate through the gel. Gel electrophoresis will therefore separate DNA molecules according to these sizes.

Separation due to size depends on the pore size of the gel matrix. In the case of agarose, which is already in polymer form before the gel is prepared; the pore size simply varies inversely with the agarose concentration. In the same field Homam *et al.*, 2003 observed that in order to obtain true data or true fingerprints for each primer, the migration distance of bands in the agarose gel must take enough R_m values to obtain cleared profiles. Dos-Santos *et al.*, 1994, suggested that the differences were primarily due to sampling errors rather than due to fundamental differences in

how RAPD and RFLP polymorphisms measure genetic distance. Also Shapiro and Maizel, 1969 mentioned that, by allowing the proteins to migrate a longer distance, an improvement in resolution is possible and the difference in molecular weight between two sub-units could be smaller.

This work aims to study the proper choice of electrophoretic mobility in agarose gel (greater extend or smaller extend) which will maximize the comparison between samples to give the best possible resolution of particular macromolecules.

MATERIALS AND METHODS

The samples of adult cotton whitefly, *Bemisia tabaci* (Geen.) were collected from cabbage from six areas (Fayoum, Beheria, Gharbia, Ismailia, Beni-Suef and Sohag) (Fig.1). The collected samples use to release the differences which related to biogeographic area in case greater extend and smaller extend of electrophoretic mobility in agarose gel.

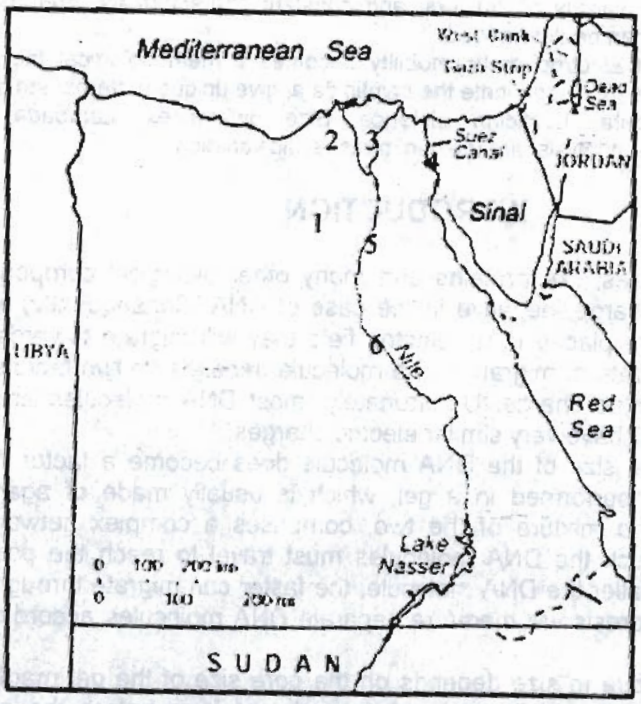


Fig.1: Map showing the distribution of tested *Bemisia tabaci* populations within Egypt

Samples were frozen at -20°C until used. DNA isolation was done according to (Sambrook *et al.*,1989).

RAPD-PCR conditions: Genomic DNA was analyzed with RAPD–PCR technique according to the method described by Williams *et al.* (1990) &Welsh and McClelland (1990).The primer used were 10-base long oligonucleotides of `arbitrary sequence, which were obtained from Operon-A primer (Operon, USA) , (OPA-1 ,OPA-7, OPA-9, OPA-10, OPA-12, OPA-14, OPA-16, OPA-17, and OPA-19). In a 50 µl final volume PCR reaction (in a 0.25 Eppendorof tube), the components of the reaction mixture for each whitefly sample were 30 ng template DNA, 2µl dNTPs (10 mM), 5µl primer (5 Pico mole µl), 5 µl $Mgcl_2$ (25 mM), 5µl Taq-polymerase buffer 10 X and 1.8 unit Taq- polymerase enzyme.

The temperature profiles were: Predenaturation at 95°C for 6 minutes and denaturation, annealing, extension were repeated 40 cycles at 94°C, 36°C, 72°C for 1,2 and 3 min., respectively. The post- extension lasted for 10 minutes at 72°C.

Agarose gel electrophoresis: 25µl of PCR products of each samples were mixed with 5 µl of blue juice loading dye (6X), loaded on 1.8% agarose gel stained with 100µl of ethidium bromide (1000X) and subjected to electrophoresis at 50V. Gel diameter was 9 cm width x12cm length. For 1/3 the length in case smaller extend [i.e] small relative mobility) and 2/3 the length in case of greater extend [i.e] long relative mobility). The PCR products were visualized on UV transilluminator. Both types of smaller &greater extend are photographed at the same focus by using Polaroid MP4 camera.

Statistical analysis: Data of smaller extend, greater extend and suitable R_m (i.e) greater extend in case of strong amplification + smaller extend in case of poor amplification were analyzed by binary scoring (i.e., presence/absence) The data were analyzed by using 27 types of cluster analysis: Squared Euclidean distance, Eculidan distance, Size difference, Pattern difference, Variance, Dispersion, Shape, Simple matching, Phi4-point correlation, Lambada, Anderberg,s D, Dice, Hamann, Jaccard, Kulczynski 1, Kulcynski 2, Lance and Williams, Ochiai, rogers and Tanimato, Russel and Rao, Sokal and Sneath 1, Sokal and Sneath2, Sokal and Sneath 3, Sokal and Sneath4, Sokal and Sneath5, Yule,sY & Yule,sQ (SPSS Program , Virgin 12).

RESULT AND DISCUSSION

In the present paper, the genetic ship among the tested populations of *B.tabaci* within Egypt is not discussed, we did this at length PCR fingerprint using the methodology of RAPD-PCR. The work was restricted in the present paper to study the parameter (condition) at which the relative mobility of bands are chosen according to the state of amplification. On the other hand, this work is an extent ion to previous work (Homam *et al.*, 2003).

A- Parameters to obtain the best possible resolution of particular macromolecules in agarose gel electrophoresis.

The separation of double-strand linear nucleic acid fragments is entirely based on size, since the charge density is the same for pieces of different

length. The same is true for single-strand nucleic acid fragments under denaturing conditions. Conformational differences can be used to resolve polynucleotides under special circumstances. The obtained results indicated that the following are the special circumstances

I- At emerge of strong amplification of PCR product and intense fragments from agarose well to gel matrix; this condition need to greater extend (long R_m). At greater extend the sieving process occurs at series different pore size for different molecular size of DNA fragments. At specific pore size sedimentate fragments characterized with the same molecular size, and so on, this gives the best possible resolution of particular macromolecules. OPA -1, 9 , 10 , 14 , 16 , & 19 give 39 ,44 ,45 ,37 ,44 & 37 bands in case of smaller extend (small R_m), but in case of greater extend (long R_m) give 62, 68, 53, 44, 61& 61 bands (Table,1). For optimal resolution, all fragments interest should be retarded by the gel, but not completely excluded. When the size range of the sample components is too wide to be sieved by a gel of single pore size the long R_m value must be done.

II- At emerge poor amplification of PCR product and faint fragments from agarose well to gel matrix, this condition needs to smaller extend because at long R_m the faint fragments diffuse and disappear. At OPA - 17, all the samples had faint fragments except the sample of Gharbia had a single intense and distinct fragment. The samples are characterized with faint PCR fragments, the number of fragments decrease at long R_m (Table, 1). At the single sample which characterized with a single intense and distinct fragment, the number of fragments increased from 7 to 9 fragments at long R_m. Also OPA-12 gave 42 fragments at smaller extend but gave 38 bands at greater extend. The suitable parameter of electrophoretic mobility is greater than small extend and great extend by 87 and 13 bands (Table, 1).

Any RAPD bands of mixed intensity (i.e. strong, faint, fuzzy or sharp) are generated with each primer, because the target DNA is undefined, one or more copies of the target DNA may exist per genome or the percentage of annealing of primer to target DNA may vary. The problem of mixed intensity bands provokes the well known sensitivity of PCR (Bielawski *et al.*, 1995). Surzycki (1986) mentioned in general, without determine the condition of choosing the R_m., that, the electrophoresis should be continued until the bands of interest migrate 1/2 to 2/3 the length of the gel. To obtain maximum resolution of many bands, electrophoresis should be continued until tracking dye (for example, bromophenol blue) has moved 70%to 80%the length of the gel.

Statistical analysis using analysis of variance and Duncan multiple range test grouped the tested *B. tabaci* populations into one group in the case of smaller extend. (i.e) gave non significant differences among the tested *B. tabaci* populations. The analysis in both cases, greater extends and suitable parameter of electrophoretic mobility showed that, Sohag populations were significantly different from Beheira. But populations of Ismailia, Beni-Suef, Gharbia and Fayoum had overlapping results with each other ($P. < 0.05$) (Table 2).

The analysis of the data became sibling at the small R_m value as occurred when Dancan analysis was used. The data was sibled and all the tested population gave non significant differences. This view is opposite the truth and lead to deviation in the results. When the long R_m value is found, the data clearly appeared and the tested populations ranked to different groups. Homam *et al.*, (2003), stated that in order to obtain true data or true fingerprints for each primer, the migration distance of bands in the agarose gel must take enough R_m values to obtain cleared profiles. When the R_m values are small, crowded and hardly discriminated bands are obtained .Crowded bands or broad bands can be resolved into well defined bands when allowing enough R_m values. In the same trend Shapiro and Maizel (1969) mentioned that, by allowing the proteins to migrate a longer distance, an improvement in resolution is possible and the difference in molecular weight between two sub-units could be smaller.

B-Cluster analysis

The Cluster analysis has been successfully implemented for studying of populations and ecology. These studies have involved the clustering of organisms or populations into groups from particular hosts or environments (Bridge 1993).

I-small extend

The obtained data are listed in Table (3). All used types of cluster analysis are similar in grouping the tested samples except Russel and Rao & Euclidian distance analysis. Similar cluster analysis grouped the tested samples into two main clusters (Fig.2). The first cluster included whitefly populations collected from Fayoum & Gharbia. On the other hand, the second cluster was divided into two subgroups. The first subgroup included whitefly populations from Beheria & Ismalia, while the second subgroup included whitefly populations from Bin- Suef & Sohag.

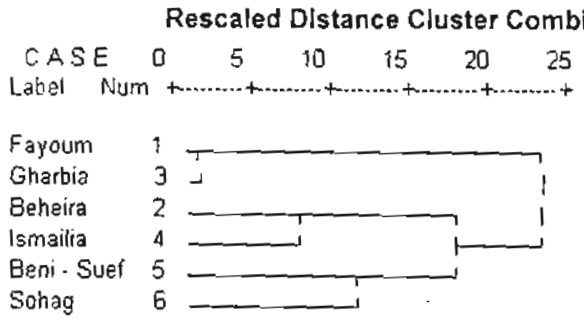


Fig. 2: Dendrogram using Average Linkage (Between Groups)

Russel and Rao cluster analysis grouped the tested samples into two main clusters (Fig.3). The first cluster included whitefly populations collected from Fayoum & Gharbia. On the other hand, the second cluster was divided into two subgroups. The first subgroup included two sites. The first site included whitefly populations from Beni- Suef& Sohag, while the second site included Ismailia, while the second subgroup included whitefly population from Beheira.

Table (1): The total number of RAPD-PCR fragments generated by a battery of 9 primers and their relative amplifications efficiency.

Primer codes	Smaller extend						Total	Greater extend						Total	Amplification efficiency
	1	2	3	4	5	6		1	2	3	4	5	6		
OPA – 1	6	6	7	5	6	7	37	9	9	11	12	8	12	61	Strong
OPA – 7	8	7	9	7	5	9	45	7	6	7	7	8	9	44	Faint
OPA – 9	6	7	4	8	8	11	44	9	7	6	14	16	16	68	Strong
OPA – 10	6	10	8	8	5	8	45	9	12	9	10	7	6	53	Strong
OPA – 12	7	6	8	5	6	7	39	9	9	11	11	10	12	62	Strong
OPA – 14	7	5	7	8	7	8	42	6	6	7	7	6	6	38	Faint
OPA – 16	7	6	8	8	9	6	44	6	6	11	8	8	10	49	Strong
OPA – 17	3	4	7	6	6	4	30	2	4	9	3	1	2	21	Faint
OPA – 19	6	3	6	11	11	7	44	9	2	10	10	15	15	61	Strong
Total	56	54	64	66	63	67	370	66	61	81	82	79	88	457	

1-6 represents samples of whitefly collected from Fayoum, Beheira, Gharbia, Ismailia, Beni-Suef and Sohag governorates, respectively.

Table (2): Duncan analysis for the three cases

Tested samples	Smaller Extend			Greater Extend			Suitable Parameter		
	N	Mean	Duncan Grouping	N	Mean	Duncan Grouping	N	Mean	Duncan Grouping
Beheira	129	0.41860	a	192	0.31250	c	193	0.32124	c
Fayoum	129	0.43411	a	192	0.33854	bc	193	0.35233	bc
Gharbia	129	0.49612	a	192	0.41667	ab	193	0.41969	abc
Beni-Suef	129	0.48837	a	192	0.41667	ab	193	0.42487	abc
Ismailia	129	0.52713	a	192	0.43229	ab	193	0.44041	ab
Sohag	129	0.51938	a	192	0.46354	a	193	0.47150	a

Mean followed with the same letters are not significantly different ($P<0.05$)

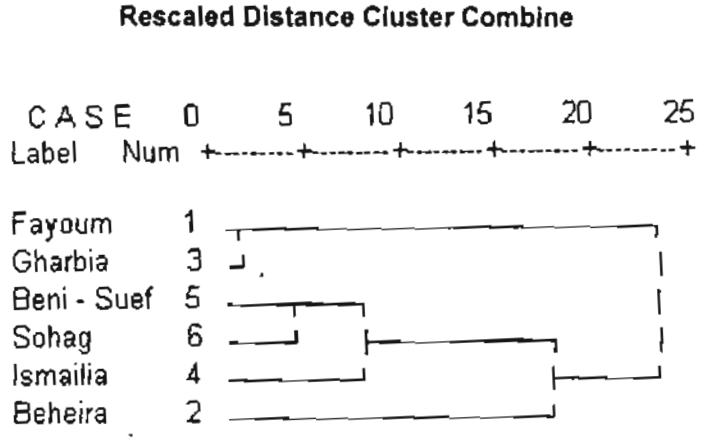


Fig.3: Russel and Rao analysis showed Dendrogram using Average Linkage (Between Groups)

Eculidan distance analysis grouped the tested populations into four clusters (Fig.4). The first cluster was divided into two subgroups. The first subgroup included whitefly populations of Gharbia & Beni-Suef, while the second subgroup included population of Beheira. The second cluster represented Sohag population. The third cluster represented Fayoum population. The fourth cluster represented Ismailia population.

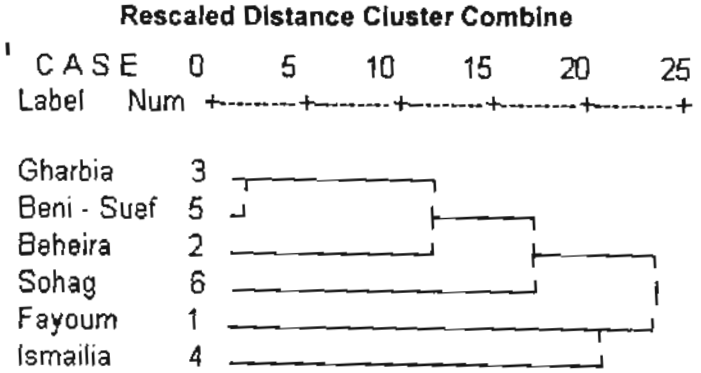


Fig.4: Eculidian distance analysis showed Dendrogram using Average Linkage (Within Groups)

II-greater extend

Dendrogram of 27 types of cluster analysis are listed in table (2). Dendrogram of greater extend included Fig.2, 5, 6, and 7. Cluster analysis represented in Fig.5 grouped the tested populations into two main clusters. The first cluster was divided into two subgroups. The first subgroup included whitefly populations of Beni-Suef and Sohag while the second subgroup included whitefly population of Ismailia. Also, the second cluster was divided

into two subgroups. The first subgroup included whitefly populations collected from Fayoum and Gharbia, while the second subgroup represents Beheira population.

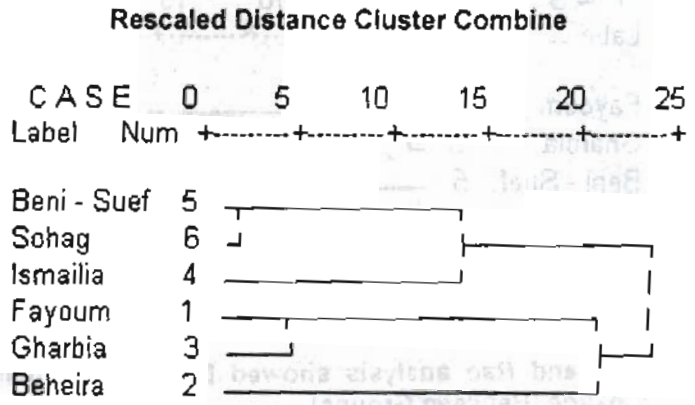


Fig.5: Dendrogram using Average Linkage (Between Groups).

While size differences cluster analysis grouped the tested populations into two main clusters (Fig.6). The first cluster was divided into two main subgroups. The first subgroup included Gharbia, Beni -suef and Ismailia populations. While the second subgroup represented Sohag population. On the other hand, the second cluster included Fayoum and Beheira.

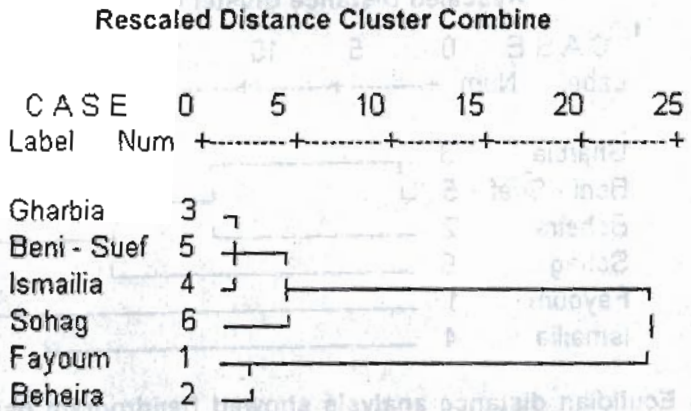


Fig.6: Size Differences analysis showed Dendrogram using Average Linkage (Between Groups).

Eculidan distance cluster analysis (Fig.7) grouped the tested populations into two main clusters. The first cluster was divided into two subgroups. The first subgroup included whitefly populations of Ghariba & Sohag, while the second subgroup represented whitefly population of Ismailia. Also the second cluster was divided into two subgroups. The first

subgroup included whitefly populations of Beheira & Beni-Suef, while the second subgroup represented whitefly population of Fayoum.

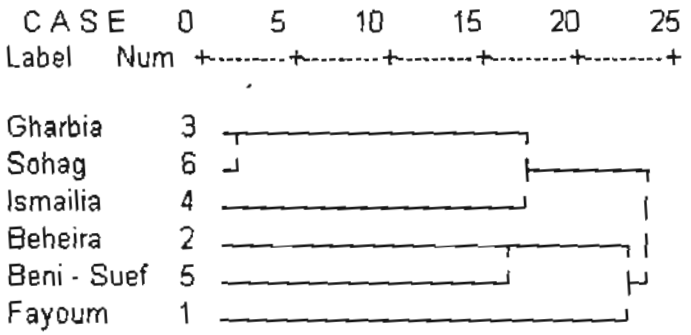


Fig.7: Eculdian distance analysis showed Dendrogram using Average Linkage (Between Groups).

III-Suitable parameter of electrophoresis fragments.

Maximizing the number of fragments by greater extend in case of strong amplification and keeping of the stability of the number of fragments by smaller extend in case of poor amplification is the suitable choosing for R_m. of RAPD-PCR fragments. The previous condition will increase the characters (units) which used in Operational Taxonomic Units (OTUs) to give more quantity and quality differences among variable. Additional analysis can then be undertaken on the dendrogram to assess the distinctness and validity of clusters, and constant characters for later use in diagnostic schemes can be determined.

Data of suitable parameter were analyzed by using 27 types of cluster analysis, listed in table (2) and shown in Figures 2, 5, 8, 9 and 10.

Eculidian distance analysis (Fig.8) grouped the tested populations into two main clusters. The first cluster was divided into two subgroups. The first subgroup was divided into two sites. The first site included populations of Gharibia and Sohag, but the second site represented the population of Beheira. The second subgroup represented the Ismailia population. The second cluster included the populations of Fayoum and Beni-Suef.

Size difference cluster analysis (Fig.9) grouped the tested samples into two main clusters. The first cluster was divided into two subgroups. The first subgroup was divided into two sites. The first site included whitefly populations of Beheira & Sohag, while the second site included whitefly populations of Fayoum & Ismailia. While the second subgroup represented Beni-Suef population. On The other hand, the second cluster represented whitefly population collected from Gharbia.

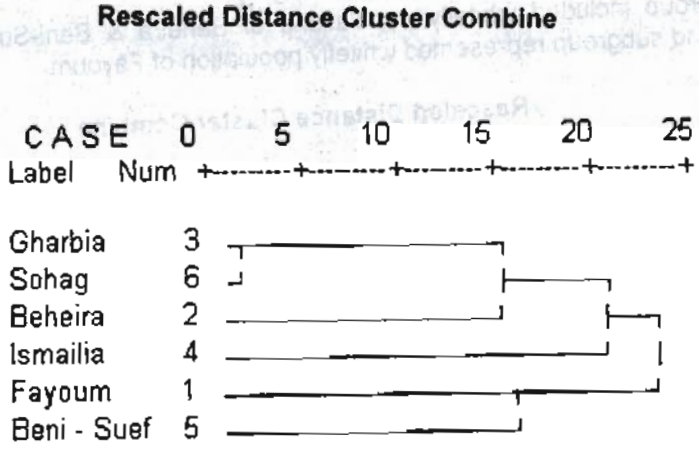


Fig.8: Eculidian Distance analysis showed Dendrogram using Average Linkage (Between Groups)

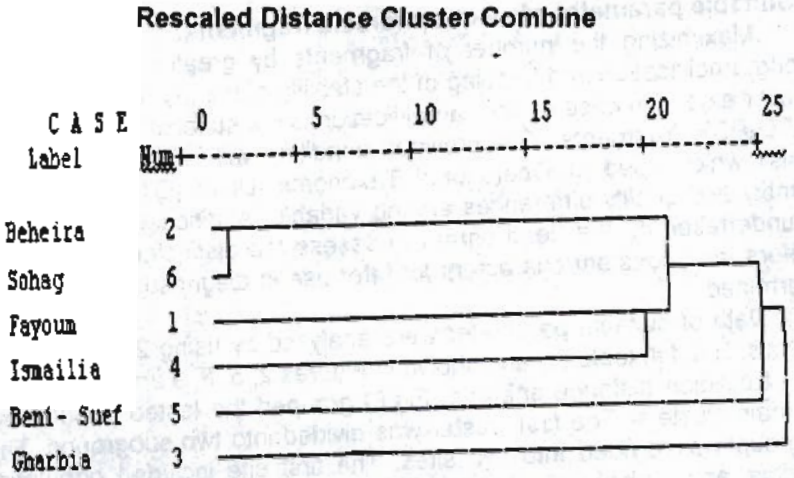


Fig.9: Size Differences analysis showed Dendrogram using Average Linkage (Between Groups)

Lambada and Anderberg's D cluster analysis (Fig.10) grouped the tested samples into two main clusters. The first cluster was divided into two subgroups. The first subgroup included whitefly populations of Beni- Suef & Sohag, while the second subgroup included whitefly populations of Beheira & Ismailia. On the other hand, the second cluster included whitefly populations collected from Fayoum & Gharbia.

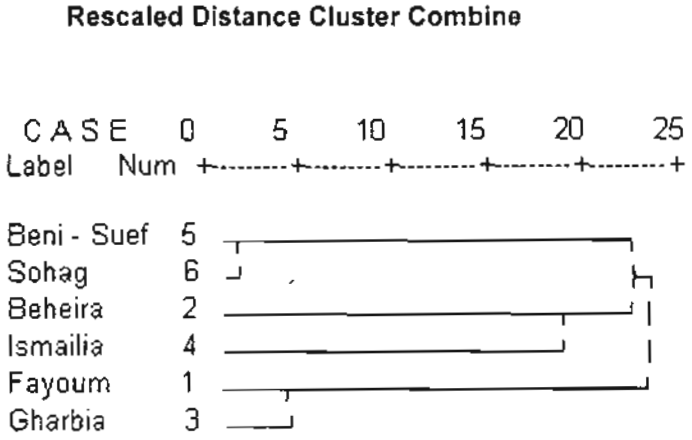


Fig.10: Lamabada and Anderberg'sD analysis showed Dendrogram using Average Linkage (Between Groups).

Results of tested cluster analysis in case of suitable parameter except four were run in common with the result of greater extend; because most primers used characterized with strong amplification (greater extend) and only two primers characterized with faint amplification (smaller extend). The obtained data indicated that, the ratio between strong amplification (greater extend) and faint amplification (smaller extend) play important role in direction the results.

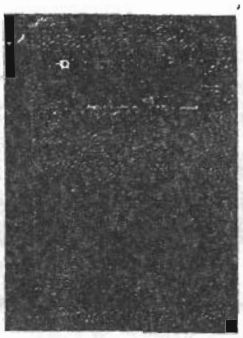
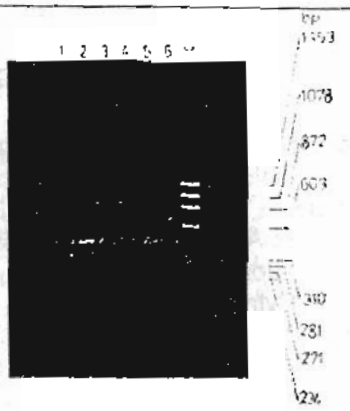
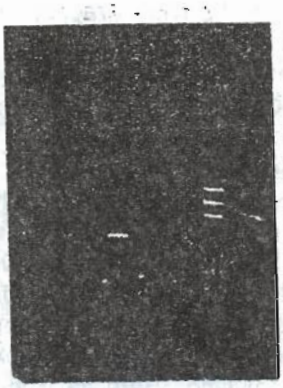
It was found that the pattern of similarities between the tested populations showed broad agreement across the small R_m values in case of strong amplification and emerged PCR product from well to gel matrix but level of discrimination was increased according to increased R_m values. By maximizing the R_m values the position and number of bands changed. Consequently, change in similarity among samples, which play role in change in data. Exploition of genetic variability has the major importance in basic genetic studies. Compiling the information gained from suitable extend could lead to more definitive results. Only Eculidian distance, Size differences, Lambada and Anderberg'sD showed a high responsible for change in input data for this reason they must be used in intraspesific variation. Both Euclidean distance and size difference are either binary equivalents of association measures for continuous variables or measures of special properties of the relationship between items. In addition, both Anderberg's D (1973) and Lambada(Goodman 1978) assess the association between items as the predictability of one given the other. Also, Eculidean distance only showed separate dendrogram in the three cases (Sneath and Sokal 1973& Abbott et al., 1985) mentioned that the most widely used measure of distance in numerical taxonomy is probably the Eculidian or taxonomic distance. Amein *et al.*, 1998 addressed the question: what is the number of polymorphic bands required to obtain a suitable classification of genotypes?

Table (3): The distribution of Figures of 27 cluster analysis in the three cases (smaller extend, greater extend and Suitable parameter of electrophoresis fragments)

Types of cluster analysis	Small extend	ater extend	Suitable parameters
Squared Euclidean distance	Fig.2	Fig.2	Fig.2
Eculidan distance	Fig.4	Fig.7	Fig.9
Size difference	Fig.2	Fig.6	Fig.10
Pattern difference	Fig.2	Fig.2	Fig.2
Variance	Fig.2	Fig.2	Fig.2
Dispersion	Fig.2	Fig.2	Fig.2
Shape	Fig.2	Fig.2	Fig.2
Simple matching	Fig.2	Fig.2	Fig.2
Phi4-point correlation	Fig.2	Fig.2	Fig.2
Lambada	Fig.2	Fig.5	Fig.11
Anderberg,s D	Fig.2	Fig.5	Fig.11
Dice	Fig.2	Fig.5	Fig.5
Hamann	Fig.2	Fig.2	Fig.2
Jaccard	Fig.2	Fig.5	Fig5
Kulczynski 1	Fig.2	Fig.5	Fig.
Kulcynski 2	Fig.2	Fig.5	Fig.5
Lance and Williams	Fig.2	Fig.5	Fig.5
Ochiai	Fig.2	Fig.5	Fig.2
Rogers and Tanimato	Fig.2	Fig.2	Fig.2
Russel and Rao	Fig.3	Fig.5	Fig.5
Sokal and Sneath 1	Fig.2	Fig.2	Fig.2
Sokal and Sneath2	Fig.2	Fig.2	Fig.5
Sokal and Sneath 3	Fig.2	Fig.2	Fig.2
Sokal and Sneath4	Fig.2	Fig.2	Fig.2
Sokal and Sneath5	Fig.2	Fig.2	Fig.2
Yule,sY	Fig.2	Fig.2	Fig.5
Yule,Sq	Fig.2	Fig.2	Fig.5

The authors concluded that the stable relation ship among the genotype is reached when the number of polymorphic bands approached 120. The present study showed that the following cluster analysis: Squared Euclidan distance, Pattern difference, Variance, Dispersion, Shape, Simple matching, Phi4-point correlation, Hamann, Rogers and Tanimato, Sokal and Sneath 1, Sokal and Sneath 3, Sokal and Sneath4& Sokal and Sneath5 not affected by the numbers of bands (129, 192 and 193 in case of small extend, greater extend and suitable parameter, respectively, of electrophoresis fragments). The previous analysis could be used in interspesific variation.

The binary scoring (i.e., presence/absence) was used in the present study which is most frequently used in RAPD-PCR investigations (Yu and Pauls 1995). In addition, some investigators have used multistate scoring to recognize differences in band intensity among samples (Strongman and Mackay 1993, Wolf and Peters-Van Rijn 1993, Howell and Newbury 1994, Weising *et al.*, 1995). Suitable R_m. help in identification the bands as missing, weak or strong (scored as 0,1, or 2, respectively).The using 3-state system to score reproductive band intensity variation to account for allele concentrations differences in polled samples(Hadrys *et al.*,1993, Tinker *et al.*,1994).Hence, Maximizing the number of fragments by greater extend in case strong amplification and keeping of the stability of the number of fragments by smaller extend in case of poor amplification is the suitable choosing for R_m. of RAPD-PCR fragments, give the true value of similarity among the tested samples, discriminate the cryptic data, facilitate to give unique pattern, use in intraspecific variation and avoid the deviation in data.

Smaller extend	Greater extend
OPA – 16	
	
OPA – 1	
	
OPA - 17	

Panel (1): Showed RAPD-PCR fragments in both smaller and greater extend cases.

CONCLUSIONS

The choice of small extend of PCR fragments for poor amplification, faint or diffuse fragments and long extend of PCR fragments for strong amplification, intense and distinct fragments in gel matrix, when emerge from agarose well become a methodological factor to obtain optimal resolution. The right choice led to discriminate the cryptic data, give unique patterns and avoid the deviation in data. Eculidian distance, Size differences, Lambada and Anderberg'sD cluster analysis are used in intraspesific variation.

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تأثير الحركة النسبية لقطع RAPD-PCR في انحراف النتائج

همام بخيت همام

معهد بحوث وقاية النباتات، شارع نادي الصيد – الدقي – الجيزة – مصر

النتائج أعطت دليل علي أن الاختيار الرئيسي لحركة الفرد الكهربي (امتداد كبير أو امتداد صغير) لقطع PCR علي النحو التالي:-

١ – في حالة بزوغ تضخم قوي وقطع كثيفة لمنتجPCR من أجا روس well إلي بقية الاجاروس، هذه الظروف تحتاج إلي امتداد كبير

٢ – في حالة بزوغ تضخم ضعيف وقطع باهته لمنتجPCR من أجا روس well إلي بقيه الاجاروس هذه الظروف تحتاج إلي امتداد صغير

بزيادة عدد قطعRAPD-PCR في الحالة الأولى وثبات عدد القطع فى الحالة الثانية يُمكن من أعطاء اختلافا أكثر كما و نوعاً بين المتغيرات المختبرة. وهناك تحاليل إضافية يمكن أجرائها في Dendrogram لإثبات صحة التحليل العنقودي و الصفات التي اعتمد عليها لاستخدامها لاحقا في برنامج (Scheme) التميز.

اختيار حركة الفرد الكهربي لقطع PCR أصبح عاملا مؤثرا في طريقة العمل لكي نحصل علي أعلي صورة واضحة و تميز البيانات المستترة و أعطاء نموذج فريد في البصمه الوراثيه، وتجنب الانحراف في النتائج، طريقة التحليل العنقودي بواسطة الاكليدان دايمستمس، زيز دهفرنسيس، لامبادا و أندربيرجس د ويمكن استخدامهم في حالة الاختلاقات البينية(intraspecific varation) .