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Standardization and Optimization of RAPD assay for genetic analysis of Noctuid species

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ABSTRACT
The genomic DNA of various noctuid species belonging to the family Noctuidae has been subjected to RAPD-PCR analysis. The main objective under study was to standardize the methods to generate RAPD markers. Regarding standardization 100 bp ladder is used, amount of DNA template to be used is 100 ng/100 µl. Twenty random primers viz. OPA 1 to OPA 20 were employed to generate RAPD markers out of which OPA13 showed better results, different RAPD profiles were observed for the different species indicating that the RAPD-PCR analysis can be applied as a useful tool in quickly screening the strains to aid in discriminating these species. Finally, regarding the standardization of PCR product and loading dye the combination of 20 µl of PCR product with 4 µl of dye produced very refined bands with OPA 13. The potential for RAPD-PCR technique is to provide useful genetic data for the discrimination up to the inter-specific and intra-specific level of noctuid species is of concern and is hereby discussed.

Keywords: genomic DNA, noctuid, primers, RAPD-PCR, standardization.

1. Introduction
The family Noctuidae commonly known as Owlet moths is one of the largest families in the order Lepidoptera which comprises more than 35,000 known species placed in 29 sub-families and 4,200 genera. The noctuid moths can be easily recognized from other lepidopterans by their prominent eyes and comparatively robust structure. The noctuids mostly have drab forewings, although some have brightly coloured hind wings. Noctuids are a prominent group of terrestrial insect fauna and food web, including innumerable ecological and economically important species [13]. DNA markers are used to provide raw information, based on which an ecologist makes estimates of genetic diversity and gene flow between species [2]. Molecular data provides the means to differentiate sympatric species from allopatric and parapatric species, and modes of evolution [4]. This issue may be overcome by using the polymerase chain reaction-based randomly amplified polymorphic DNA (RAPD-PCR) which does not require previous knowledge of a DNA sequence to design primers [17, 18]. This technique has generated useful results in the differentiation between insect species and strains [10]. This method has a great potential for the identification of DNA polymorphism in the genome quickly and efficiently [8]. They may also be useful to differentiate between species with very close morphological characters e.g. at the larval stage [9] as well as between closely related strains of some species [15]. Despite all these advantages important questions have been raised for a practical application. One of the most important is the reproducibility of the results obtained with the same primers across a variety of protocols and laboratory conditions and varying level of searched polymorphism (inter-specific, intra-specific, between strains, etc.). Because the most frequent cause of poor quality RAPD is poor quality DNA, it is important to purify DNA with methods that will yield pure and undegraded DNA. With the ready-to-use kits available for genomic cells and tissue DNA extraction followed by ready-to-go RAPD analysis systems the quality and reproducibility of PCR-RAPD analysis may be greatly improved. However, the reproducibility in the banding pattern discriminating among species or strains, when studied with several individuals in a pool or with a single individual has not been thoroughly investigated with these kits. In the current study, we used commercial kits to determine the polymorphism among species, on a pool of several individuals or on the genomic DNA extracted from a single individual. Our study was aimed to standardize the RAPD-PCR technique in view of the optimization of the experiment for creating a band profiling pattern of the noctuid moths.

2. Materials and methods

2.1 DNA isolation

The different noctuid moth species used in this study were collected by light trap and killed with ethyl vapours. In the first experiment, the RAPD-PCR was standardized using various DNA isolation kits such as Qiagen's DNeasy^{R} Blood & Tissue Kit, G biosciences and Macherey-Nagel nucleospin tissue kit. Finally the protocol of Macherey-Nagel nucleospin tissue kit was used as the other two kits were not successful in isolating DNA of the noctuid moths. The moth was ground in liquid nitrogen and not more than 50 mg of moth powder was introduced into 1.5 ml Eppendorf tube. Then, 25 µl of protienase K and buffer T1 180 µl were added for complete lysis and tubes were incubated at 56 ^{0}C for 2-3 hours. To the solution added 200 µl of buffer B3, followed by thorough vortexing. The tubes were then incubated in water bath at 70 ^{0}C for 10-15 minutes. Insoluble particles were visible and centrifugation was done at 14000 rpm for 2 minutes, the supernatant was transferred to a new Eppendorf tube. Then 210 µl of ethanol was added and contents were thoroughly mixed by vortexing. The mixture was transferred into a mini spin column placed in a 2 mL collection tube. The tubes were centrifuged for 1 min at 11,000 g. The mini spin column was placed in a new 2 mL collection tube and 500 µL washing buffer BW was added before centrifugation for 1 min at 11000 g. Then, mini spin column was placed in a new 2 mL collection tube. 600 µL buffer B5 was added and the tube was centrifuged for 1 min at 11,000 g to dry the membrane. Again, centrifuge the column for one minute at 11000 rpm in order to remove excessive ethanol. For the DNA elution step, after the mini spin column was transferred with caution in a 1.5 mL new tube, 100 µL eluting buffer BE was added directly onto the membrane before incubating at room temperature for 2 min followed by centrifugation for 1 min at 6,000 g. This elution step was repeated once in the same conditions. The tubes with re-hydrated DNA were stored at 4 °C in a refrigerator before PCR.

2.2 RAPD-PCR primers

The choice of discriminating primers was done among the twenty primers

Primer	Sequence 5'->3'	Tm Value ^{O}C	GC Content %
OPA1	CAGGCCCTTC	34	70
OPA2	TGCCGAGCTG	34	70
OPA3	AGTCAGCCAC	32	60
OPA4	AATCGGGCTG	32	60
OPA5	AGGGGTCTTG	32	60
OPA6	GGTCCCTGAC	34	70
OPA7	GAAACGGGTG	32	60
OPA8	GTGACGTAGG	32	60
OPA9	GGGTAACGCC	34	70
OPA10	GTGATCGCAG	32	60
OPA11	CAATCGCCGT	32	60
OPA12	TCGGCGATAG	32	60
OPA13	CAGCACCCAC	34	70
OPA14	TCTGTGCTGGC	32	60
OPA15	TTCCGAACCC	32	60
OPA16	AGCCAGCGAA	32	60
OPA17	GACCGCTTGT	32	60
OPA18	AGGTGACCGT	32	60
OPA19	CAAACGTCGG	32	60
OPA20	GTTGCGATCC	32	60

2.3 PCR protocol

For preparing master mix the following products were included: distilled water 13.8 µL; 2.5 µL Taq buffer; 2.5 µL dNTP's; 2 µL primer (OPA-13); 0.4 µL of Taq polymerase. All the steps are to be kept under 0-4 ^{0}C. To each reaction mixture add 4 µL of insect DNA extract and 21 µL master mix. The samples were placed in the thermal cycler using the following PCR conditions: one cycle 95 °C, 5 min; 55 cycles 72 °C, 2 min 30 sec.; one cycle 72 °C, 10 min; one cycle 4 °C, 5 min.

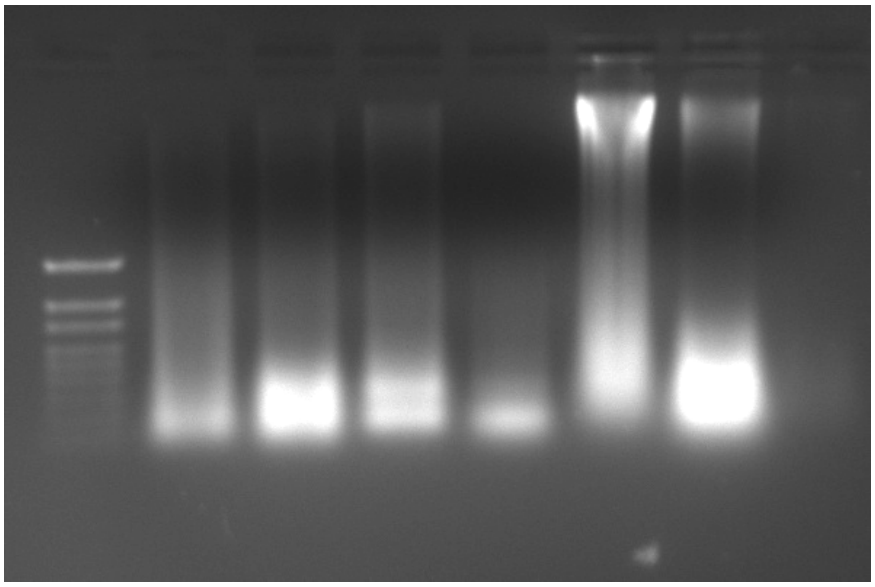
2.4 Gel analysis

The electrophoresis revealing the banding pattern of the RAPD-PCR amplified DNA was carried out on a 2% agarose gel using 1x TBE buffer containing 7 µl ethidium dibromide. In each well of the gel, 20 µL of PCR amplified product and 4 µL of 6x loading buffer were loaded. The electrophoresis was performed until good separation of RAPD bands and the capacity of the electrophoresis system (e.g. 150 V for3 h).

3. Results and Discussion

3.1 Standardization of DNA ladder

For standardization of DNA ladder 3 different species were studied. Each species had its own specific DNA pattern allowing clear discrimination with another species. Markers of 100 bp and 1 kb size were used for analysis. RAPD products revealed that 100 bp marker showed better results.

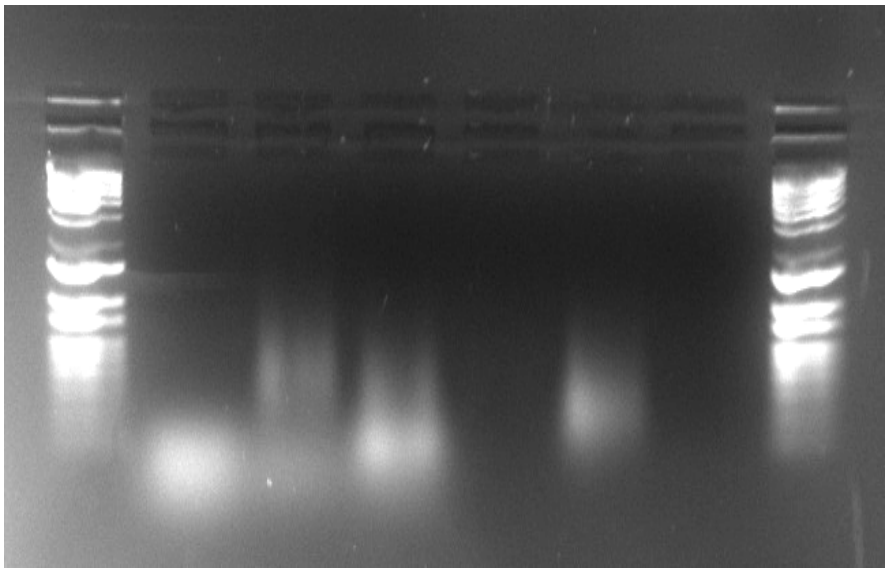


Lane 0: 100bp marker

Lane 1: ***Athetis delecta*** (m) Lane 4: ***Athetis delecta*** (m)

Lane 2: ***Athetis delecta*** (f) Lane 5: ***Spirama retorta*** (m)

Lane 3: ***Athetis delecta*** (f) Lane 6: ***Cerura litura*** (f)



Lane 0 & 7: 1000 bp universal marker

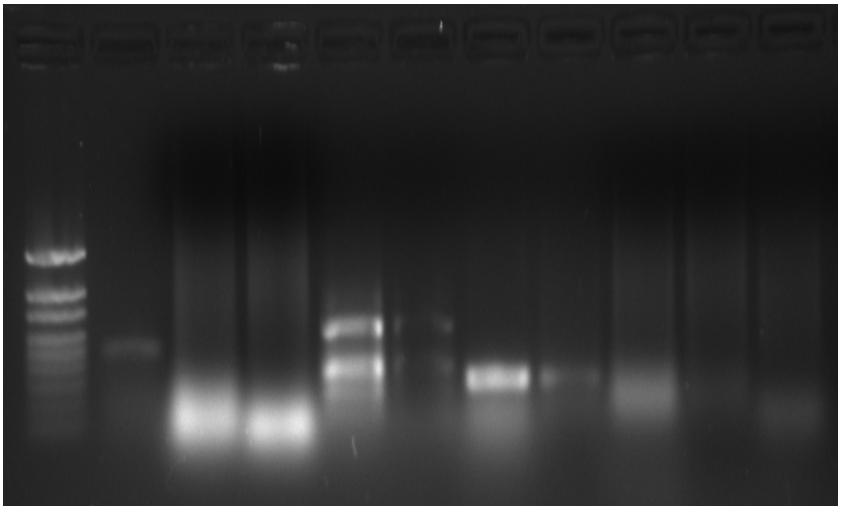
Lane 1: ***Agrotis ipsilon*** (OPA-6) Lane 4: ***Agrotis ispilon*** (OPA-7)

Lane 2: ***Diarsia consanguinea*** (OPA-6) Lane 5: ***Diarsia consanguinea*** (OPA-7)

Lane 3: ***Pseudaletia separata*** (OPA-6) Lane 6: ***Pseudaletia separata*** (OPA-7)

3.2 Optimization of the quantity of DNA template

Amount of DNA template to be used is 100 ng/100 µl during RAPD-PCR process, hence proving as a vital tool of information for the quantification and standardizing the RAPD-PCR process to be used for establishing the genetic difference between the species. Also the purity of DNA and DNA amount affects the efficiency of derived bands. The primers can increase the DNA amount in a sample as they are annealed randomly in RAPD analysis; however, at that point, the DNA quality and amount used are important. Good quality, pure and DNAase free buffers should be used to prevent contaminations. It is suitable to add RNAase for the purpose of preventing RNA interaction following the DNA isolation. High DNA concentration may prevent DNA amplification [16, 11, 1, 14, 5].

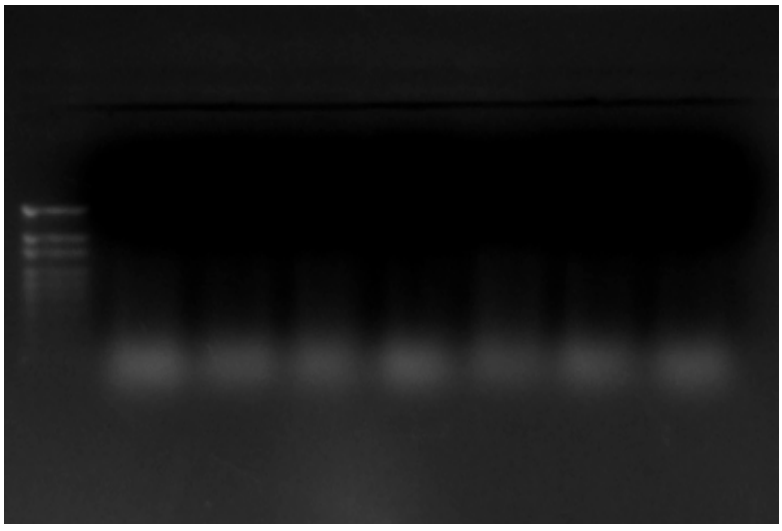


Lane 0 : 100 bp marker
Lane 1: ***Sarrothripa grisea*** (OPA-6) 50 ng/100 µl
Lane 2: ***Sarrothripa grisea*** (OPA-6) 150 ng/100 µl
Lane 3: ***Sarrothripa grisea*** (OPA-6) 200 ng/100 µl
Lane 4: ***Sarrothripa grisea*** (OPA-6) 100 ng/100 µl
Lane 5: ***Sarrothripa grisea*** (OPA-6) 150 ng/100 µl
Lane 6: ***Sarrothripa grisea*** (OPA-7) 50 ng/100 µl.
Lane 7: ***Sarrothripa grisea*** (OPA-7) 150 ng/100 µl
Lane 8: ***Sarrothripa grisea*** (OPA-7) 200 ng/100 µl
Lane 9: ***Sarrothripa grisea*** (OPA-7) 100 ng/100 µl
Lane 10: ***Sarrothripa grisea*** (OPA-7) 150 ng/100 µl

3.3 Standardization of primers

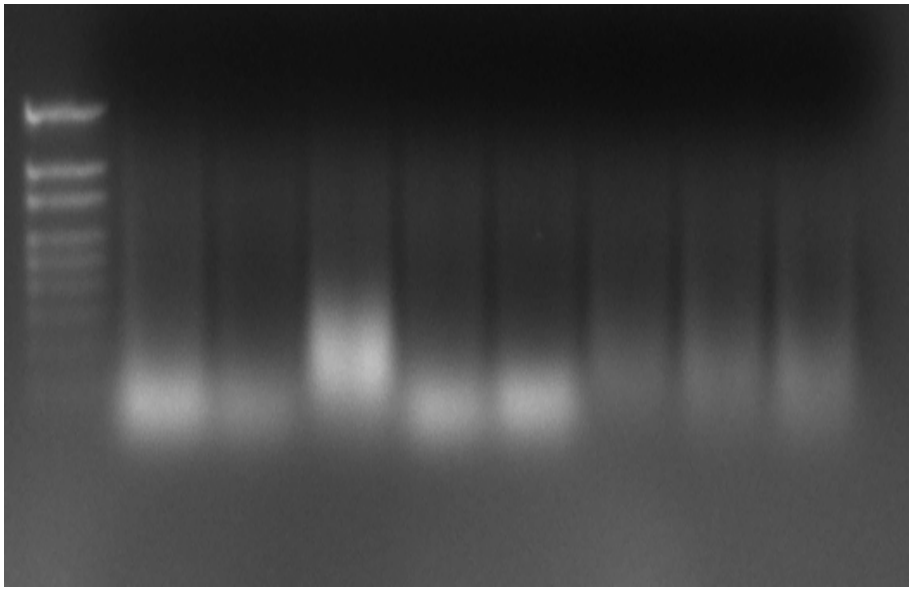
For this purpose twenty OPERON series primers viz. OPA1 to OPA20 were used and similarly twenty reaction mixtures were made. To prevent the anomaly only one noctuid species viz. ***Athetis delecta*** (m) was taken in agar gel 1and 2, while in agar gel 3 & 4 male and female of ***Athetis delecta*** were taken. Four agar gels were made, agar gel 1 and agar gel 2 containing primers having Tm value 32 ^{0}C and GC content of 60% (viz. agar gel 1 was loaded with primers OPA3,4,5,7,8,10, and OPA 11; agar gel 2 was loaded with primers OPA 12, 14, 15, 16, 17, 18, 19 and OPA20). Meanwhile agar gel 3 and agar gel 4 containing primers having Tm value 34 ^{0}C and GC content of 70% (viz. agar gel 3 loaded with primers OPA 9 and OPA 13; agar gel 4 was loaded with primers OPA 1, 2 and OPA 6). Out of all 20 primers, only OPA 9 and OPA 13 showed bands and the species had same profile band with both the primers, the only difference was OPA-13 produced sharper and more bands than OPA-9. Increasing the primer concentration in PCR mix results in a decrease in the number of wide bands; however, the number of small particles would increase. It is determined as a result of assays on 0.2, 0.5 and 1 µmol primer concentrations that although more wide bands were observed in 0.2 µmol concentrations, the number of bands with small molecular weight increased in 1 µmol concentration. However, excess amount of primers in the setting causes an increase in nonspecific products; on the other hand there occurs a decrease in the efficiency of bands, because the primer would be depleted rapidly within PCR cycles in low concentration. Therefore, it is important to determine the best concentration in order to ensure the efficient of band its reproducibility and clarity [16, 11, 1, 14, 5]. It is considered that 1 µmol primer concentration is suitable for olive samples.

1) Banding patterns in gel -1 with different primers:



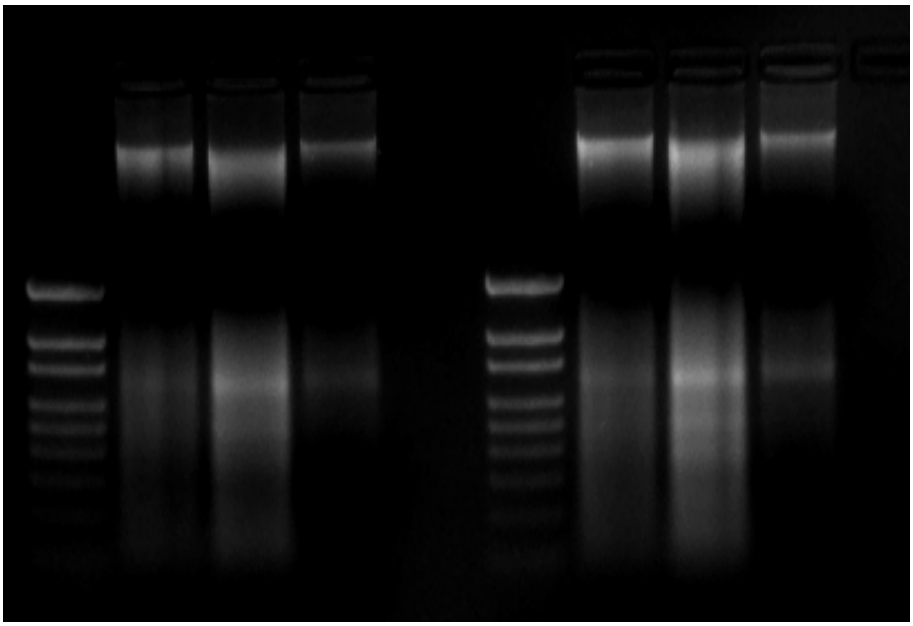
Lane 1 : 100 bp marker, Lane 2 : OPA 3 Lane 3 : OPA 4 Lane 4 : OPA 5 Lane 5 : OPA 7 Lane 6 : OPA 8, Lane 7 : OPA 10 Lane 8 : OPA 11

2) Banding patterns in gel -2 with different primers



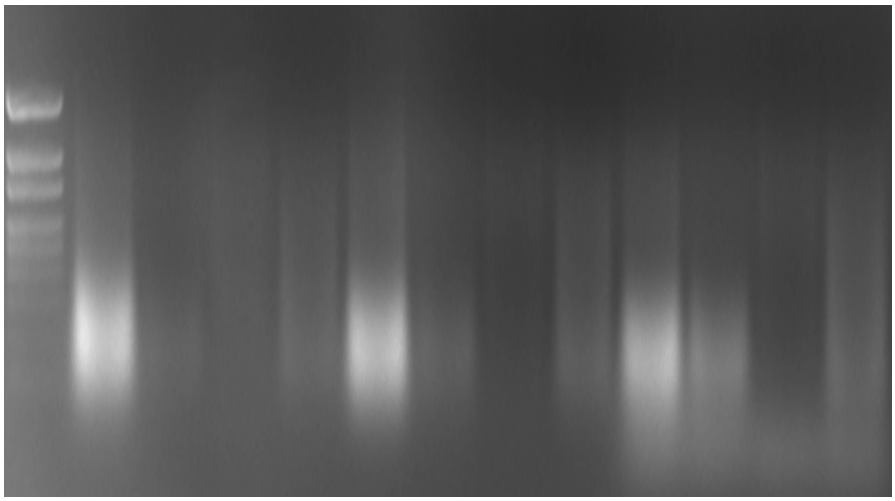
Lane 1 : 100 bp marker
Lane 2 : OPA 12
Lane 3 : OPA 14
Lane 4 : OPA 15
Lane 5 : OPA 16
Lane 6 : OPA 17
Lane 7 : OPA 18
Lane 8 : OPA 19
Lane 9 : OPA 20

3) Banding patterns in gel -3 with different primers



Lane 1 : 100 bp marker
Lane 2 : OPA 9 + ***Athetis delecta*** (m)
Lane 3 : OPA 9+ ***Athetis delecta*** (f)
Lane 4 : OPA 9 + ***Athetis delecta*** (m)
Lane 5 : empty lane
Lane 6 : 100 bp marker
Lane 7 : OPA 13 + ***Athtlis delecta*** (m)
Lane 8 : OPA 13 + ***Athelts delecta*** (f)
Lane 9 : OPA 13 + ***Athetis delecta*** (m)

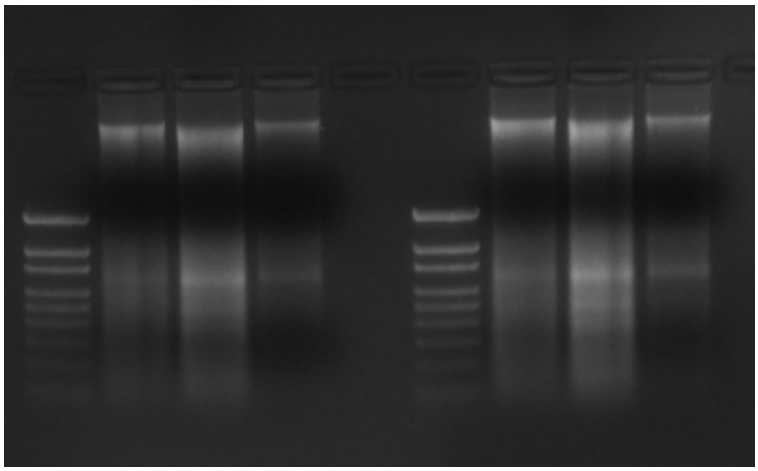
4) Banding patterns in gel - 4 with different primers



Lane 1 : 100 bp marker
Lane 2 : OPA 1 + ***Athelis delecta*** (m)
Lane 3 : OPA 1+ ***Athelis delecta*** (f)
Lane 4 : OPA 1 + ***Athelis delecta*** (m)
Lane 5 : OPA 2+ ***Athelis delecta*** (m)
Lane 6 : OPA 2 + ***Athelis delecta*** (m)
Lane 7 : OPA 2 + ***Athelis delecta*** (f)
Lane 8 : OPA 2 + ***Athelis delecta*** (m)
Lane 9 : OPA 6 + ***Athelis delecta*** (m)
Lane 10 : OPA 6 + ***Athelis delecta*** (f)
Lane 11 : OPA 6 + ***Athelis delecta*** (m)

3.4 Standardization of PCR product and loading dye

Athetis delecta (f) was used as an insect source, operon primers OPA 9 & OPA 13 were used since they proved to be the excellent in template binding and DNA amplification. Current studies were carried out keeping in view amount of the dye to be used in proportion of the DNA template for their expression in gel electrophoresis. As we know that template DNA obtained from DNA extraction kit should be of the value of 100 ng/µl, so the attempts were taken to obtain the same. Dye used here was 0.25% bromophenol blue, 0.25% xylene cyanol; 60% glycerol (6X). DNA quantity after amplification used was 4 µl, 20 µl & 2 µl. while the dye used was in the corresponding ratio of DNA quantity taken i.e. 20 µl, 4 µl & 22 µl, since we know that the given dye is 6X and we had to make it 1X before the electrophoresis can be done. In lane 8 the combination of 20 µl of PCR Product with 4 µl of dye produced very refined bands with OPA 13 than any other lanes with different dye to PCR product ratio. The RAPD-PCR technique has been reported as an effective method to discriminate geographically and genetically isolated populations [11].



All species are *Athetis delecta* (f), d = dye, PP = PCR product
Lane 1 : 100 bp marker
Lane 2 : OPA 9 + AD+ 4 µlpp +20 µld
Lane 3 : OPA 9 + AD+ 20 µlpp +4 µld
Lane 4 : OPA 9 + AD+ 2 µlpp +22 µld
Lane 5 : Blank
Lane 6 : 100 bp marker
Lane 7 : OPA13 + AD+ 4 µlpp +20 µld
Lane 8 : OPA 13 + AD+ 20 µlpp +4 µld
Lane 9 : OPA 13 + AD+ 2 µlpp +22 µld

Majority of RAPD studies are based on determining the clustering by applying the cluster analysis on the derived bands and thus determining the genetic similarities and distances. The evaluation capability of bands and non-formation of primer artifacts are dependent on the sensitivity of PCR conditions. Suitable primer and primer concentration, purity of obtained DNA, number of

cycles and denaturation, annealing, extension periods and purity and concentration of tampons included in the reaction mix affect the RAPD analysis and correspondingly its products. Evaluation capability and reproducibility of RAPD products is dependent on PCR conditions. Therefore, it is important to determine the best concentration in order to ensure the efficient RAPD bands [6, 3, 12, 19, 7].

4. Conclusion

Our study aimed to standardize the RAPD-PCR technique in view of the optimization of the experiment for creating a band profiling pattern of the noctuid moths. The main objective under study was to standardize the methods to generate RAPD markers. Regarding standardization 100 bp ladder is used, amount of DNA template to be used is 100 ng/100µl. Twenty random primers viz. OPA 1 to OPA20 were employed to generate RAPD markers out of which OPA13 showed better results, different RAPD profiles were observed for the different species indicating that the RAPD-PCR analysis can be applied as a useful tool in quickly screening the strains to aid in discriminating these species. Finally, regarding the standardization of PCR product and loading dye the combination of 20 µl of PCR product with 4 µl of dye produced very refined bands with OPA 13.

5. Acknowledgements

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