Original Article

Mutagenicity Assessment of Organophosphates using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism Assay

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ABSTRACT

Objectives: In this study we have evaluated the mutagenicity of organophosphate pesticides acephate, chlorpyrifos, and profenofos using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay with the mosquito *Culex quinquefasciatus* taken as an experimental model. **Materials and Methods:** Second instar larvae were treated with LC_{20} of each pesticide for 24 h and mutations induced in the sequence of mitochondrial *COII* gene (690bp) were studied from restriction patterns generated with *Alu*I, *Pac*I, and *Psi*I restriction endonucleases. **Results:** Variations in the number and size of digested fragments were recorded from treated individuals compared with controls showing that the restriction enzymes created a cut at different locations. In addition, sequences of *COII* gene from control and treated individuals were also used to confirm the RFLP patterns. From the sequence alignment data, it was found that mutations caused the destruction and generation of restriction sites in the gene sequence of treated individuals. **Conclusion:** This study indicates that all the three pesticides had potential to induce mutations in the normal sequence of *COII* gene and also advocates the use of PCR-RFLP assay as an efficient, rapid, and sensitive technique to detect mutagenicity of pesticides.

Key words: Acephate, chlorpyrifos, *Cx. quinquefasciatus*, mutagenicity, PCR-RFLP, profenofos

INTRODUCTION

The use of pesticides has become indispensible in modern agricultural systems. Their application has increased food production and decreased the risk of various pests. Despite these benefits, pesticides are also known to harm nontarget organisms including human beings. Organophosphate pesticides are among the largely used classes of compounds for pest control in various scenarios. The toxicity of organophosphate pesticides is attributed specifically to the inhibition of acetylcholinesterase, an enzyme involved in regulation of neurotransmission by hydrolysis of the neurotransmitter, acetylcholine. Subsequent accumulation of acetylcholine and consequential over stimulation of acetylcholine receptors has been the chief mechanism of their acute toxicity.[1] Apart from various forms of neurotoxicity, which may or may not have relevance to their ability to inhibit acetylcholinesterase, organophosphate pesticides exposures have been associated with genotoxicity and reproductive toxicity.[2,3] Thus, continuous monitoring of the genotoxic potential of these pesticides has become mandatory. Today, a number of protocols are available for genotoxicity assessment of pesticides by using different test organisms. The logic behind the use of diverse organisms lies in the fact that there is universality in the structure and functions of deoxyribonucleic acid (DNA); therefore, genotoxic agents would affect them in the same way by

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reacting with certain sites and modifying it in number of ways such as cleavage of phosphodiester bonds, insertions, deletions, and substitutions. In relevance to this, the present polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)-based investigations were undertaken to evaluate the mutagenicity of three organophosphate pesticides acephate, chlorpyrifos, and profenofos with the mosquito *Culex quinquefasciatus* taken as an experimental model. This assay helped in measuring the extent of mutations which tend to alter a restriction endonuclease recognition sequence. It involved the PCR amplification of a specific region of DNA followed by restriction enzyme digestion of the PCR products. Mutations were detected by the loss or generation of a restriction sites which were seen in the form of variations in the number and size of restriction fragments. In the present study, a portion of mitochondrial *COII* gene was amplified from control and pesticide treated individuals which was then digested with *Alu*I, *Pac*I, and *Psi*I restriction endonucleases after which the patterns generated from control and treated individuals were compared.

As for the relevance of present work, it is pertinent to add that in the recent years there had been an increase concern toward reducing the number of higher laboratory animals for research due to ethical issues. This has lead to more emphasis on the use of alternative animal models and in reference to this the present study involved the use of mosquito *Cx. quinquefasciatus* as a test system. Although it differs from the other test organisms in terms of metabolism, DNA repair and physiological processes affecting chemical mutagenesis, yet the universality of DNA and the genetic code provides a rationale to predict the mutagenicity of mutagens. In this context, flies have been found to be equally as sensitive to toxicants as mammals because some studies have shown that flies and mammals have a similar dose-response relationship.[4-6]

MATERIALS AND METHODS

Test chemicals

For the present study, technical-grade acephate (75% SP), chlorpyrifos (40% EC), and profenofos (50% EC) manufactured by Scientific Fertilizers Co. Pvt. Ltd., Coimbatore, India, were used. In order to assess the toxicity of a chemical, it is always crucial to determine a suitable dose for its effective action in the test system. Accordingly, LC_{20} was found to be an ideal concentration and the LC_{20} values for acephate, chlorpyrifos, and profenofos as calculated by probit analysis were 5 µg/mL, 3.46 µl/mL, and 5.19µl/mL, respectively.

Test organism

Cx. quinquefasciatus Say, used as an experimental insect for the present investigations, was collected from the cattle sheds in the early morning from the village inhabitations near Chandigarh. The gravid females were held in the test tubes, where they were allowed to oviposit on a strip of wet filter paper. A larval colony was raised from these eggs in a BOD incubator by feeding the stocks with a diet consisting of finely powdered dog biscuits and yeast tablets.[7,8] The chemical treatment was given to the second instar larvae for which they were kept in standardized dose of the pesticide for 24 h after which they were transferred to pesticide-free water for further growth up to adult stages. Freshly hatched unfed adults were stored in separate Eppendorf tubes at −20°C for DNA extraction.

Amplification

The DNA was extracted from adult mosquitoes by following the protocol of Ausubel *et al.*[9] according to which each specimen of freshly hatched unfed adult was homogenized. A portion of *COII* gene was amplified using forward and reverse primers *viz*: 5'-AGATTTTATCTTTTGTTAGAA-3' and 5'-TTGCTTTCAGTCATCATCTAATG-3'.[10] PCR amplification was performed in a 25 µL reaction volume containing 0.2 mM dNTP mix, 1 × buffer, 1mM $MgCl_2$, 1U Taq polymerase, 0.2 µM primers, and 2 µL of DNA template. The amplification reactions were performed as described by Williams *et al.*,[11] according to which, 25 µL of reaction mixture was loaded in a thermocycler which was programmed for the initial one cycle for denaturation of DNA at 94°C for 10 min followed by 35 cycles each of denaturation, annealing of primer, and extension of DNA at 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min, respectively, terminating with a final extension at 72°C of 5 min. In all such amplifications, a negative control consisting of all the components of reaction mixture except the DNA was also carried out to rule out the experimental errors. The PCR products and DNA ladder were electrophorased on 2% agarose gel containing ethidium bromide and visualized on ultraviolet transilluminator. These amplified products were sequenced and the DNA sequences were aligned using ClustalW multiple sequence alignment program.

Restriction digestion

After amplification, 4 µL PCR product was digested with sufficient units of selected restriction enzyme (*Alu*I, *Pac*I, or *Psi*I) in 2 µL of buffer for 5 h at 37°C. Reactions were terminated by incubation at 70°C for 15 min after which the digested fragments were resolved on 2% agarose gel with ethidium bromide staining and photographed on ultraviolet transilluminator.

RESULTS AND DISCUSSION

In the present PCR-RFLP analysis, *COII* amplicons from both control and treated stocks were digested with all the three restriction endonucleases and the resulting digested PCR products were isolated by using 2% agarose gel. The DNA band patterns generated from control and treated individuals were compared. This was followed by *in silico* restriction enzyme analysis of *COII* gene sequences with NEBcutter software for validation of results. This helped in obtaining the actual fragment number and fragment size. The fragment size of each fragment obtained from NEBcutter and those observed experimentally showed congruency in the results. The only difference encountered in some cases was the lack of one very small fragment that was difficult to discern on agarose gels. In addition, sequences of *COII* gene from control and treated individuals were used to confirm the RFLP pattern. The RFLP pattern generated from nontreated *Cx. quinquefasciatus COII* amplicon indicates that there was no restriction site for *Pac*I, one nicking site for *Alu*I which resulted in the production of two fragments of 604 and 86 bp. Due to its smaller size, the 86 bp band was not visible on 2% agarose gel. *Psi*I had two sites in *COII* gene sequence that yielded three bands of 350, 243, and 97 bp. The *COII* amplicon of acephate treated individual remained undigested after action of *Alu*I, because restriction site was destroyed by a mutation that changed guanine to cytosine (G → C) at base 604. Digestion with *Psi*I produced two bands of 440 and 255 bp as both the restriction sites previously present in the normal sequence were destroyed due to substitution at 99 and 343 bp and a new site was generated by another substitution at 260 bp [Table 1, Figures 1a, 1b, and 2]. The *COII* amplicon of chlorpyrifos treated specimen, remained undigested with *Alu*I because restriction site for this enzyme was destroyed by a mutation which replaced thymine with adenine (T → A) at base 606. As for the action of *Pac*I, two bands of 552 and 114 bp were produced due to the creation of a new restriction site as a transition of cytosine to thymine (C → T) took place at base 553. *Psi*I yielded two bands of 484 and 212 bp as both the restriction sites present in the normal sequence were destroyed in the treated individual due to two point mutations at 100 and 340 bp and a new site was created by another mutation at base 217 [Table 1, Figures 1c-e, and 3]. *Alu*I restriction digests of *COII* amplicon from profenofos treated individual presented no mutation except for a minor change in the length of the DNA fragments because of sequence rearrangement which occurred due to deletion of bases 4, 17, and 18. *Psi*I action yielded two fragments of 597 and 94 bp as against control in which *Psi*I generated three bands because one of the restriction sites was destroyed by a mutation at base 340 [Table 1, Figures 1f, 1g, to 4].

This investigation has shown that all the three pesticides induced mutations which were evident from the variations

Table 1: PCR-RFLP product sizes of *COII* gene sequence of control and treated *Culex quinquefasciatus*

Type of sample	PCR product size (bp)	PCR-RFLP product size (bp)		
		*Alu*I	*Pac*I	*Psi*I
Control	690	604, 86	690*	350, 243, 97
Acephate treated	695	695*	695*	440, 255
Chlorpyrifos treated	696	696*	552, 144	484, 212
Profenofos treated	695	601, 90	695*	597, 94

*PCR product not digested (no restriction site)

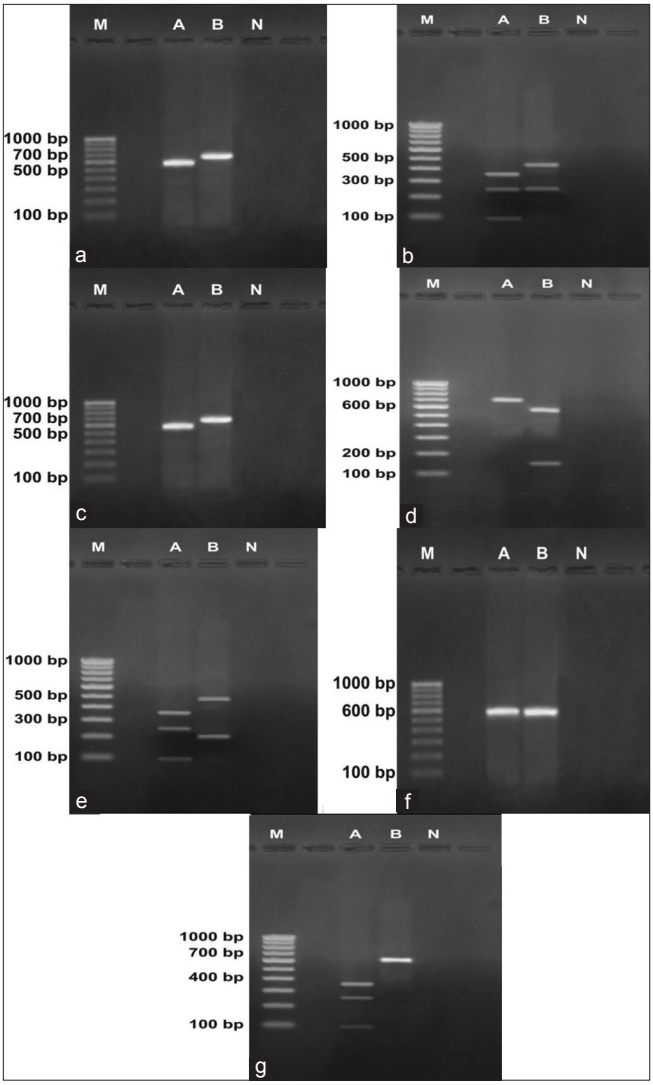

Figure 1: RFLP patterns obtained after: *Alu*I (a) and *Psi*I (b) digestion of the *COII* amplicon of control and acephate treated *Culex. quinquefasciatus*; *Alu*I (c), *Pac*I (d) and *Psi*I (e) digestion of the *COII* amplicon of control and chlorpyrifos treated *Culex quinquefasciatus*; *Alu*I (f) and *Psi*I (g) digestion of the *COII* amplicon of control and profenofos treated *Culex quinquefasciatus.* Lane M: Gene ruler, Lane A: RFLP pattern from control individual, Lane B: RFLP pattern from treated individual, Lane N: Negative control

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CLUSTAL 2.1 multiple sequence alignment

CONTROL         TTATGGCACCTGAGCAATTTAGGATTACAAAATAGTTCTTCTCCATTAATAGAACAATTA 60
TREATED         TTGTGGC--CTGAGCAATT-AAGATTACAAAATAGTTCTTCTCCATTAAAAGAACAATTA 57
                ** ****  ********** * ************************** **********

CONTROL         AATTTTTTTCATGATCATACAGTTTTAATTTTAATTATAATTACAGTAATAATTACTTAT 120
TREATED         AAATTTTTTCATGATTATACAGTTTTAAATTTATTTATTATTACAGTAATAATTACTTAT 117
                ** ************ ************ **** **** ********************

CONTROL         GTAATAGGTATATTATTTTTCAATAAATTTACAAATCGATATTTATTACATGGACAAACT 180
TREATED         GTAATAGGTACATTATTTTTCAATAAATTTACAAATCCATATTTACTACATGGACAAACT 177
                ********** ************************** ******* **************

CONTROL         ATTGAAATCATTTGAACAATTCTTCCTGCAATTATTTTAATATTTATTGCTTTTCCATCA 240
TREATED         ATTGAAATCATTTTAACAATTCTTCCTGCAATTATTTTAATATTTATTGCTTTGCCATCA 237
                ************* ************************************** ******

CONTROL         CTTCGGTTATTATATTTATTAGATGAAATTAATTCTCCTTTAATTACTTTAAAGGCTATT 300
TREATED         CATCGGTTATTATATTTATAAGATGAAATTAATTCTCCTTTAATTACTTTAAAGGCTATT 297
                * ***************** ****************************************

CONTROL         GGACATCAATGATACTGAAGTTATGAATATTCTAATTTTATAATTTAGAATTTGATTCA 360
TREATED         GGACATCAATGATACTGAAGTTATGAATATTCTAATTTTATATATTTAAAATTTGATTCA 357
                **************************************** ***** ***********

CONTROL         TATATAATTCCAACAAATGAATTAGATTTAAATGGATTCCGATTATTAGATGTTGATAAT 420
TREATED         TATATAATTCCAAAAAATGAATTCGATTTAAATGGATGCCGATTATTAGATGTTGATAAT 417
                ************* ********* ************* **********************

CONTROL         CGAATTATTTTACCATTAAATAATCAAATTCGAATTTTAGTAACTGCTACTGATGTTCTT 480
TREATED         CGAATTATTTTACCATTAAATAATCAAATTCGGATTTTAGTAACTGCTACTGATGTTCTT 477
                ******************************** ****************************

CONTROL         CACTCATGAACAGTTCCTTCTTTAGGAGTAAAAATTGATGCTACTCCAGGCCGATTAAAT 540
TREATED         CACTCATGAACAGGTCCTTCTTAAGGAGTAAAAATTGATGGTACTCCAGGCCGATTTAAT 537
                ************* ******** ***************** *************** ***

CONTROL         CAAACTAATTTTCTAATTAATCAATCTGGTCTTTTTTTTGGACAATGTTCTGAAATCTGT 600
TREATED         CAAACTAATTTTCCAATTAATCAATCTGGTCTTTTTTTTGGATAATGTTCTGAAATCTTT 597
                ************* **************************** *************** *

CONTROL         GGAGCTAATCATAGTTTTATACCTATTGTTATTGAAAGAATTCCAATAAATTATTTTATT 660
TREATED         GGACCTAATCATAGTTTTATACCCATTGTTATTGAAAGAATTCCAATAAATTATTTTATT 657
                *** ******************* ************************************

CONTROL         AAATGAGTTTCTTCTCAATTAAT---CTAGATG----- 690
TREATED         AAATGAGTTTCTTCTCAATTAATGCATTAGATGACCGA 695
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Figure 2: Restriction sites of *AluI* (AGCT) and *PsiI* (TTATAA) in *COII* gene sequences of control and acephate treated *Culex quinquefasciatus*

in the restriction pattern of treated individuals from controls. These differences resulted from base substitutions, insertions, deletions, or sequence rearrangements within the restriction enzyme recognition sequences. From the sequence alignment data, it was found that mutations caused the destruction and generation of restriction sites in the *COII* gene sequence of treated individuals. The presence of undigested DNA fragments indicated that a mutation had destroyed a restriction site previously present in the normal sequence. When a mutation generated a new restriction site, the sequence was cleaved by the specific restriction endonuclease, while the normal sequence remained unaltered. This event could be easily detected from the number and size of DNA bands separated after gel electrophoresis.

Studies carried out so far on the mutational activity of acephate, chlorpyrifos, and profenofos have shown that these pesticides were able to induce a variety of changes in the genomic integrity of the affected individuals. For example, acephate has been reported to increase the incidence of intercalary heterochromatic linkages in the polytene chromosomes of treated larvae of *Anopheles subpictus*.[12] A significant increase in sister chromatid exchange along with the decreased mitotic index in human peripheral lymphocytes was also observed.[13] The studies carried out on chlorpyrifos showed that it increased the frequency of apoptosis in *Drosophila melanogaster*[5] and mean comet tail length in leucocytes cells of mice.[14] It also caused dose-dependent increase in DNA damage in the liver and brain cells of rats.[15] Profenofos has been reported to induce different types of chromosomal aberrations in the germ cells of mice.[16] It also induced apoptosis, necrosis, chromatid breaks, and single-strand breaks in cultured human peripheral blood lymphocytes.[17]

Results obtained from the present experimental work and studies carried out so far have shown that acephate, chlorpyrifos, and profenofos have significant potential as

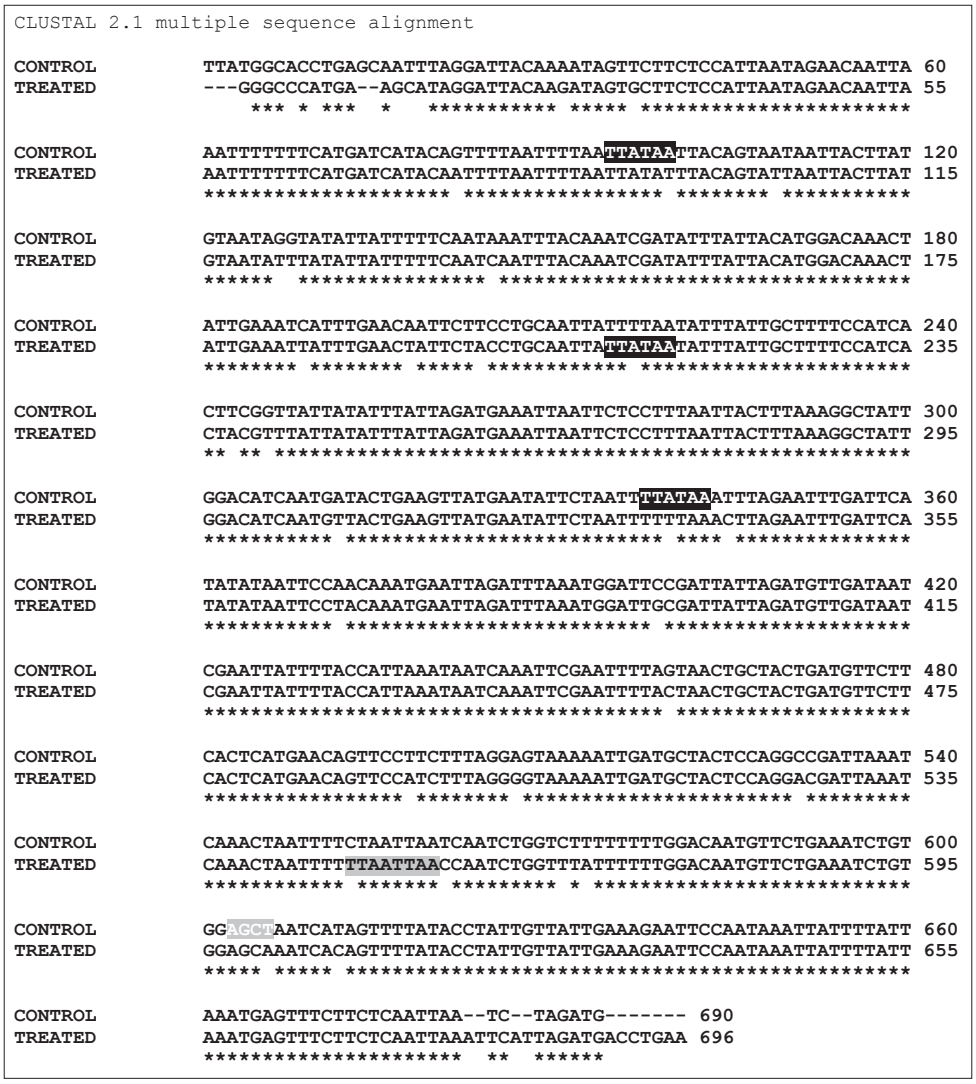

Figure 3: Restriction sites of *Alu*I (AGCT), *Pac*I (TTAATTAA) and *Psi*I (TTATAA) in *COII* gene sequences of control and chlorpyrifos treated *Culex quinquefasciatus*

DNA damaging agents. It is known that organophosphate pesticides are alkylating agents and alkylation of DNA bases either directly or indirectly via protein alkylation is involved in DNA disintegration. The phosphorous moiety in organophosphates acts as a good substrate for nucleophilic attack leading to DNA damage. As for the possible action of pesticides, it is claimed that most of these chemical formulations significantly increase the cellular reactive oxygen species, leading to modifications in the DNA in the form of base pair errors and strand breaks.[18-20]

CONCLUSION

Findings of this study indicated that acephate, chlorpyrifos, and profenofos could induce mutations in living organisms. PCR-RFLP assay can be used in combination with other tests for screening mutagenic effect of chemicals and for investigating the implications of DNA damage that can provide information at molecular level which may be used to determine the potential of a chemical to induce carcinogenicity. This study advocates the use of PCR-RFLP assay as an efficient, rapid, and sensitive technique for detection of mutagenicity caused by chemicals and also suggest that pesticides should be used judiciously and carefully.

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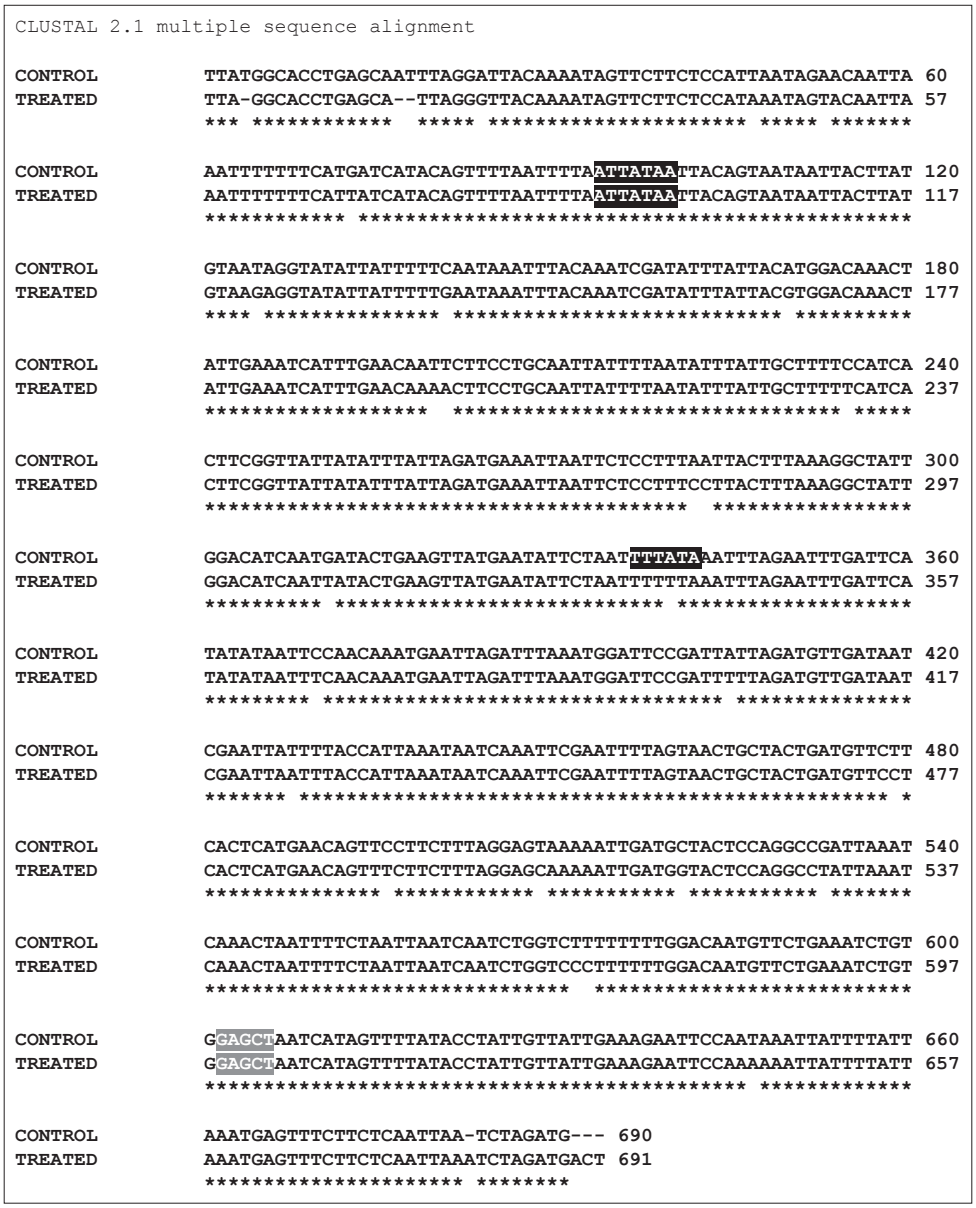

Figure 4: Restriction sites of *Alu*I (AGCT) and *Psi*I (TTATAA) in *COII* gene sequences of control and profenofos treated *Culex quinquefasciatus*

REFERENCES

1. Costa LG. Current issues in organophosphate toxicology. Clin Chim Acta 2006;366:1-13.
2. Shadnia S, Azizi E, Hosseini R, Khoei S, Fouladdel S, Pajoumand A, *et al*. Evaluation of oxidative stress and genotoxicity in organophosphorus insecticide formulators. Hum Exp Toxicol 2005;24:439-45.
3. Joshi SC, Mathur R, Gulati N. Testicular toxicity of chlorpyrifos (an organophosphate pesticide) in albino rats. Toxicol Ind Health 2007;23:439-44.
4. Siddique HR, Chowdhuri DK, Saxena DK, Dhawan A. Validation of *Drosophila melanogaster* as an *in vivo* model for genotoxicity assessment using modified alkaline Comet assay. Mutagenesis 2005;20:285-90.
5. Gupta SC, Mishra M, Sharma A, Deepak Balaji TG, Kumar R, Mishra RK, *et al*. Chlorpyrifos induces apoptosis and DNA damage in *Drosophila* through generation of reactive oxygen species. Ecotoxicol Environ Saf 2010;73:1415-23.
6. Mishra N, Tewari RR. Cytotoxic and genotoxic effects of mercury in house fly *Musca domestica* (Diptera: Muscidae). Cell Mol Biol 2011;57:122-8.
7. Singh KRP, Patterson RS, La-Brecque GC, Razdan RK. Mass rearing of *Culex pipiens fatigans* Weid. J Com Dis 1975;7:31-53.
8. Clements AN. The biology of mosquitoes. London: Chapman and Hall; 1996.
9. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, *et al*. Short protocols in molecular biology. 4th ed. New York: John-Wiley and Sons; 1999.
10. Ho CM, Liu YM, Wei YH, Hu ST. Gene for cytochrome c oxidase subunit II in mitochondrial DNA of *Culex quinquefasciatus* and *Aedes aegypti* (Diptera: Culicidae). J Med Entomol 1995;32:174-80.
11. Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV. DNA

polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 1990;18:6531-5.

12. Chaudhry A, Anand PK, Geeta, Singh S, Lovleen. Ectopic pairing of the intercalary heterochromatin in the organophosphate pesticide treated mosquito chromosomes (Culcidae: Diptera). Cytologia 2006;71:431-7.
13. Ozkan D, Yuzbasioglu D, Unal F, Yilmaz S, Aksoy H. Evaluation of the cytogenetic damage induced by the organophosphorus insecticide acephate. Cytotechnology 2009;59:73-80.
14. Rahman MF, Mahboob M, Danadevi K, Saleha Banu B, Grover P. Assessment of genotoxic effects of chlorpyriphos and acephate by the comet assay in mice leucocytes. Mutat Res 2002;516:139-47.
15. Mehta A, Verma RS, Srivastava N. Chlorpyrifos-induced DNA damage in rat liver and brain. Environ Mol Mutagen 2008;49:426-33.
16. Fahmy MA, Abdalla EF. Genotoxicity evaluation of buprofezin, petroleum oil and profenofos in somatic and germ cells of male mice. J Appl Toxicol 1998;18:301-5.
17. Prabhavathy Das G, Shaik AP, Jamil K. Cytotoxicity and genotoxicity induced by the pesticide profenofos on cultured human peripheral blood lymphocytes. Drug Chem Toxicol 2006;29:313-22.
18. Poovala VS, Kanji VK, Tachikawa H, Salahudeen AK. Role of oxidant stress and antioxidant protection in acephate-induced renal tubular cytotoxicity. Toxicol Sci 1998;46:403-9.
19. Saleha Banu B, Danadevi K, Rahman MF, Ahuja YR, Kaiser J. Genotoxic effect of monocrotophos to sentinel species using comet assay. Food Chem Toxicol 2001;39:361-6.
20. Hreljac I, Filipic M. Organophosphorous pesticides enhance the genotoxicity of benzo (a) pyrene by modulating its metabolism. Mutat Res 2009;671:84-92.

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